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EDITORS
JACQUES LOEB W. J. V. OSTERHOUT

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COMPARATIVE STUDIES ON RESPIRATION.

VII. Respiration and Antagonism.

Introductory Note.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, June 15, 1919.)

The relation of antagonism to such fundamental life processes as respiration and photosynthesis has received scant attention. The writer began studies on this subject some years ago, but owing to other interests the investigations did not progress beyond the preliminary stage. It seemed desirable that they should be carried forward, and this was facilitated by the development of new methods of measuring respiration and photosynthesis.[1]

A series of studies has recently been commenced in which these methods have been employed. Care was taken to make frequent measurements, as earlier work had shown this to be important. The fact that the rate of respiration could be determined as often as once every 3 minutes made it possible to obtain satisfactory time curves.

The plan of these investigations involves a comparative study of the antagonistic effects of salts on different kinds of organisms under precisely the same conditions. In this way it is hoped that a sound basis for the formulation of general principles may be provided.

It may be of interest to mention certain improvements in technique. When a reagent is employed which has a buffer effect it is desirable to have the same buffer action during the measurement of normal respiration as during exposure to the reagent. This may be accom-

[1] Haas, A. R. C., *Science*, 1916, xliv, 105. Osterhout, W. J. V., and Haas, A. R. C., *J. Gen. Physiol.*, 1918–19, i, 1. Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17, 171.

plished by introducing the reagent into the apparatus[2] in a separate tube, having an outlet which is flush with the inside of the stopper. When the time comes to bring the reagent into contact with the organism the tube is inverted and the current of air carries the reagent over into the tube containing the organism. In order that the height of the liquid in the latter tube may not be unduly raised it may be provided at the bottom with rubber tubing, into which, after thorough mixing, some of the liquid may be received and clamped off.

If the reagent is volatile it is necessary to substitute for it (during the measurement of normal respiration) a non-volatile liquid having the same buffer action.[3] The volatile reagent may be placed in the rubber tubing, attached to the tube containing the organisms, and clamped off until the time comes to bring it into contact with the organisms. The clamp is then opened and, after thorough mixing, the height of the liquid is adjusted as described above.

Since respiration is affected by acidity and alkalinity it is desirable to add an indicator to the liquid containing the organisms in order to follow its reaction. The color of this indicator may be affected by the color of the organism or by that of the medium, but in most cases it affords useful information regarding changes in the reaction of the liquid.

There have been few previous investigations on this subject. Warburg[4] found that the consumption of oxygen by fertilized eggs of the sea urchin (*Strongylocentrotus*) fell off when the eggs were transferred from sea water to a solution of NaCl (isotonic with sea water). This is explained by the fact, previously discovered by Loeb,[5] that NaCl causes cytolysis of the eggs. Loeb had found that this injury could be prevented by the addition of a trace of NaCN. Warburg accordingly added NaCN to the solution of NaCl and found that the amount of oxygen consumed was five times the normal. Addition of $CaCl_2$ brought it back to the normal amount.

[2] For a description of this see Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, *i*, 17.

[3] See Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxv, 237.

[4] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305; *Biochem. Z.*, 1910, xxix, 414.

[5] Loeb, J., Die chemische Entwicklungserregung des tierischen Eies, Berlin, 1909.

Loeb and Wasteneys[6] pointed out that Warburg's interpretation of his results is of doubtful value, owing to complications introduced by the addition of NaCN. In experiments on the fertilized egg of *Arbacia* Loeb and Wasteneys[6] found that there was no increase in the consumption of oxygen in a solution of NaCl (without NaCN) even when due allowance was made for the percentage of eggs killed by the action of the solution. In NaCl + KCl the eggs consumed approximately the normal amount of oxygen[7] and the addition of Ca was practically without effect.

Meyerhof[8] repeated Warburg's experiments on *Strongylocentrotus*, using a solution of NaCl (without NaCN) and making determinations after 12, 15, or 20 minutes, during which short periods little injury occurs. He found that the consumption of oxygen was from two to four times as great as in sea water. The addition of $CaCl_2$ reduced the consumption of oxygen to the normal. The addition of KCl to NaCl had practically no effect.

Similar experiments have apparently not been performed upon plants.

The investigations thus far completed in the writer's laboratory show a much closer agreement with the results of Loeb and Wasteneys in regard to NaCl than with those of Warburg and Meyerhof. They also show pronounced antagonism between such substances as NaCl and $CaCl_2$ in their effect on respiration.

Reports of some of these investigations will appear in the near future.

[6] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1910, xxviii, 340; 1911, xxxi, 168.

[7] The experiment lasted an hour, during which time the solution has little injurious action.

[8] Meyerhof, O., *Biochem. Z.*, 1911, xxxiii, 291.

COMPARATIVE STUDIES ON RESPIRATION.

VIII. The Respiration of Bacillus Subtilis in Relation to Antagonism.

By MATILDA MOLDENHAUER BROOKS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, June 15, 1919.)

Although the antagonistic effects of salts on certain bacteria have been studied, no attempt has been made to ascertain whether any relation exists between these effects and the respiration. The investigations described in this paper were undertaken with a view to obtaining some light on this question.

The first attempt to study antagonism in relation to bacteria was made by Lipman.[1] He used the production of NH_3 by *Bacillus subtilis* as an index of metabolism. He allowed cultures of *Bacillus subtilis* to grow over night in various salts and combinations of salts in the proportions found in sea water, and measured the production of NH_3. There was a considerable decrease in the production of NH_3 when the salts were used singly, but this did not occur when combinations of salts were made in the proportions found in sea water.

Winslow and Falk[2] have observed antagonistic effects in experiments on *Bacillus coli*. These investigators found that cultures suspended in solutions of NaCl or $CaCl_2$ were decreased in number; that higher concentrations produced sterilization of the culture; and that a combination of NaCl and $CaCl_2$ in the molecular proportions of 5 : 1 was favorable to the growth of the organism.

[1] Lipman, C. B., *Bot. Gaz.*, 1909, xlviii, 105; 1911, xlix, 41.

[2] Winslow, C.-E. A., and Falk, I. S., *Proc. Soc. Exp. Biol. and Med.*, 1918, xv, 67.

Shearer[3] has also demonstrated similar effects of salts upon the viability of meningococcus and *Bacillus coli*. He found that a combination of NaCl and $CaCl_2$ was favorable to growth, while each salt used separately produced decrease in growth.

It is evident, therefore, that antagonistic effects are to be expected in studying bacteria, and it seemed desirable to investigate these effects in relation to respiration. For this purpose the apparatus described by Osterhout[4] was employed.

The organism selected was *Bacillus subtilis*, the same strain as that used in an investigation previously reported.[5] It had originally been isolated from water and grown daily upon agar-agar. In every case an 18 hour culture, inoculated upon agar-agar with a few cc. of 0.75 per cent dextrose solution, was used. These inoculations were incubated at 37°C. and the resulting heavy growth of bacteria was washed off with 0.75 per cent dextrose solution and centrifugated to get rid of any foreign substances that may have surrounded the bacteria. The bacteria were then suspended in 0.75 per cent dextrose solution and were ready for experimentation. It is very important to use cultures not older than 24 hours, as the respiration of the older cultures is considerably diminished.

In making up the solution of dextrose and the salts, distilled water was employed. The salts used were NaCl, $CaCl_2$, and KCl in molecular concentrations from 0.05 M to 1.0 M. Experiments on $MgCl_2$, which belong to this series, are in process of completion.

The temperature varied in the course of the experiments from 18–20°C.

The indicator used was 5 drops of 0.01 per cent phenolsulfonephthalein in 10 cc. of water. Tap water was used, as distilled water has a pH value not exceeding 7.1 and this was not alkaline enough for measuring with this indicator. Buffer solutions were made from boric acid and borax, having pH values of 7.78 and 7.60 respectively. These buffers were used as the standard for comparison in determining the color change produced in the indicator as the CO_2 was driven over.

[3] Shearer, C., *Proc. Roy. Soc. London, Series B*, 1917, lxxxix, 440; *Proc. Camb. Phil. Soc.*, 1919, xix, 263.

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17, 171.

[5] Brooks, M. M., *J. Gen. Physiol.*, 1918–19, i, 193.

For experimentation, 2 cc. of the emulsion of bacteria were placed in the apparatus, and the air was caused to circulate. The CO_2 produced by the bacteria was thereby carried over into the indicator, and the reciprocal of the time required to change the color from pH 7.78 to 7.60 was taken as the rate of respiration or production of CO_2. When the indicator had reached the end-point required, the stop-cock was closed, thus allowing the CO_2 to be washed out of the system again by passing the air through the NaOH, and thereby returning the indicator to its original color. In this way, a series of readings could be taken. In general these were remarkably constant. It was found that the rate of respiration, under normal conditions (*i.e.* of bacteria placed in 0.75 per cent dextrose solution), was practically constant for about 6 hours. The experiments, however, lasted only 70 minutes. This included the time necessary for the establishment of the normal rate, usually 10 minutes, and the time for determining the effect of the salt.

When the normal rate had been determined, the salt was added and the change in the rate was observed. The first reading was discarded owing to the possibility of experimental error, as the result of CO_2 dissolved in the salt solution. In adding the salt solution, the system was opened because it was found that no appreciable error was introduced by exposing the system for a moment to contact with the air.

When the bacteria had been in contact with the salt for an hour, the respiration seemed to have reached an equilibrium, as the rate then decreased very slowly during the next few hours. This decrease was faster in the higher concentrations of the salt; the lower concentrations remained constant for hours at a time. In adding the salt, 2 cc. (of double the strength desired for experimentation) were added to 2 cc. of the bacteria in 0.75 per cent dextrose solution, so that the volume remained constant throughout all the experiments.

It is generally known that a reduced pressure of oxygen has little effect for a considerable time upon the rate of oxidation, so that the slight change in the oxygen content of the system during the experiment does not introduce an experimental error.

Fig. 1 shows the manner in which the rate of CO_2 production changes under the influence of NaCl in the concentrations of 0.15,

0.1, 0.5, 0.8, and 1.0 M. During the first 10 minutes the bacteria are under normal conditions and the curve (broken line) is horizontal. After this (at the point marked 0 on the abscissa) the salt is added.

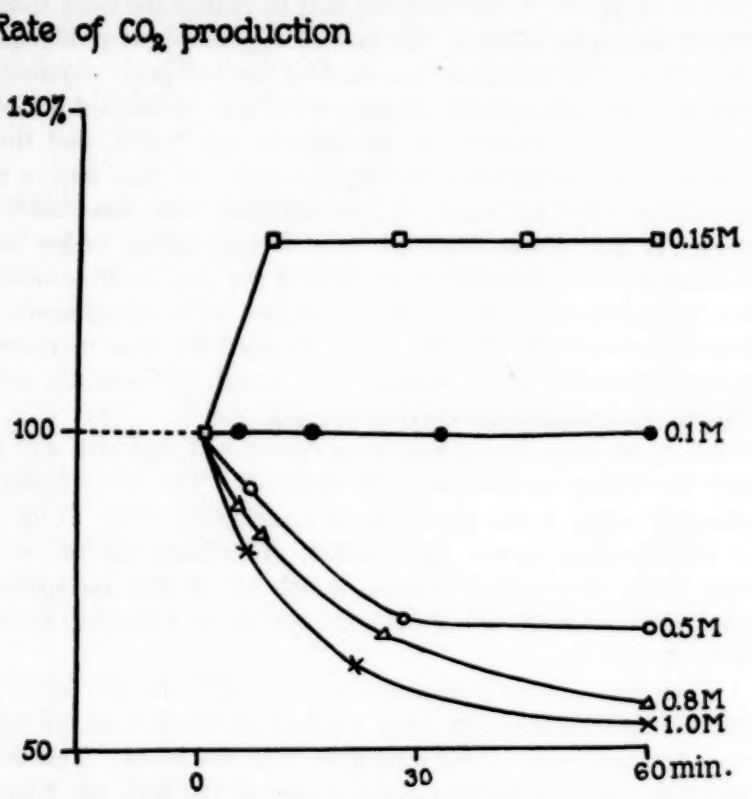

FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.15, 0.1, 0.5, 0.8, and 1.0 M NaCl. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

For example, the addition of sufficient NaCl to make the concentration 0.15 M produces a rise in the rate, which remains constant during the period of experimentation. When the concentration of NaCl

is 0.1 M the rate is normal, while in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

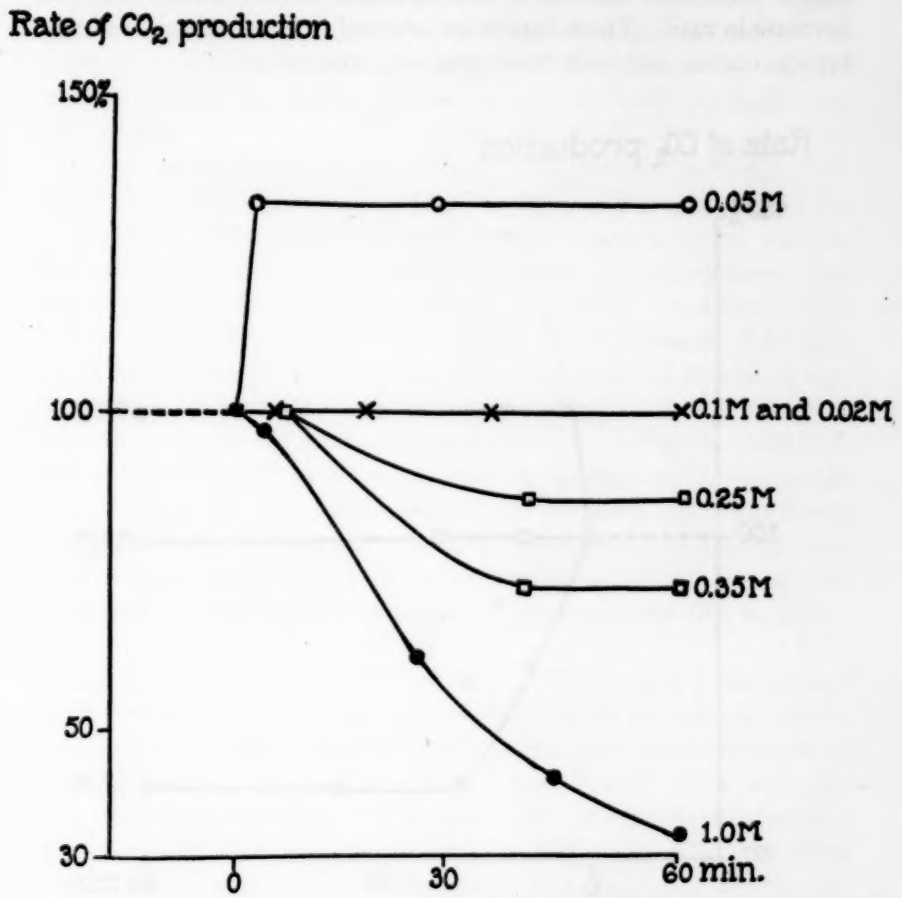

FIG. 2. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.05, 0.1, 0.25, 0.35, and 1.0 M $CaCl_2$. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

Fig. 2 shows the manner in which the rate of CO_2 production changes under the influence of $CaCl_2$ in the concentrations of 0.02, 0.05, 0.1, 0.25, 0.35, and 1.0 M. In 0.01, 0.02, and 0.1 M the rate is normal; in 0.05 M there is an increase in rate; in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

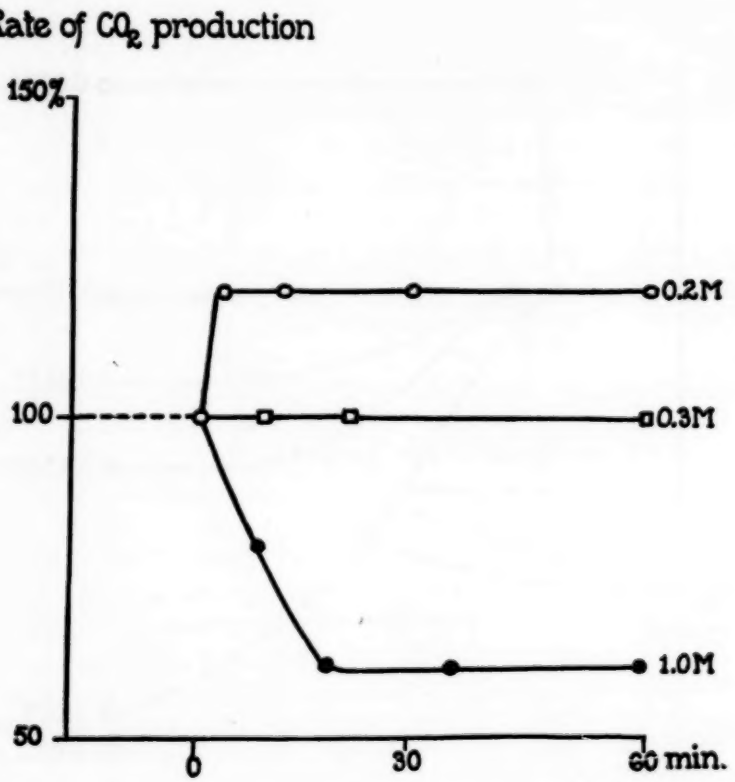

FIG. 3. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.2 M, 0.3 M, and 1.0 M KCl. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension, usually 30 seconds. Each curve represents a single typical experiment.

Fig. 3 shows the manner in which the rate of CO_2 production changes under the influence of KCl in concentrations of 0.2, 0.3, and 1.0 M. When the concentration is 0.2 M, there is an increase in the rate which remains constant for some time; in lower concentrations (0.15 and 0.1 M) the rate is normal, while in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

Fig. 4 shows the effects of various concentrations of NaCl, KCl, and $CaCl_2$ upon the rate of respiration expressed as per cent of the normal rate. The rate indicated is that produced after the bacteria had been in contact with the salt for 1 hour. The figure shows that NaCl produces an increase in the rate of respiration at a concentration of 0.15 M. In 0.5, 0.8, and 1.0 M there is a decrease. KCl produces an increase in the rate at a concentration of 0.2 M, and in concentrations higher than 0.3 M it causes a decrease. $CaCl_2$ causes an increase in the rate at a concentration of 0.05 M and in concentrations higher than 0.1 M it causes a decrease in respiration. $CaCl_2$ is the most toxic of the salts used, while KCl is the least toxic; this agrees with the results of Lipman.[1] It is of interest to note that there is evidently a correlation between the production of NH_3 of the organism (as found by Lipman) and the rate of production of CO_2 as shown here.

Fig. 5 shows the antagonism of salts. Thus, Curve A shows that when five parts of NaCl and one part of $CaCl_2$ (in the same molecular concentrations) were added to the bacteria, the rate of respiration remained normal, or as if no salt had been added. This was true only when the proportions of 5:1 were used. When other proportions were used the respiration decreased accordingly, and gave only a fraction of the normal rate. This agrees with the results of Winslow and Falk[2] on the growth of *Bacillus coli*, but not with those of Lipman,[1] on the production of NH_3 by *Bacillus subtilis*, who found no antagonism between NaCl and $CaCl_2$.

Curve B illustrates the effect of combinations of KCl and NaCl upon the rate of respiration. There are two maxima in this curve; one at 4 KCl to 6 NaCl, and the other at 6 KCl to 4 NaCl. The former is the more nearly normal, although there is no combination

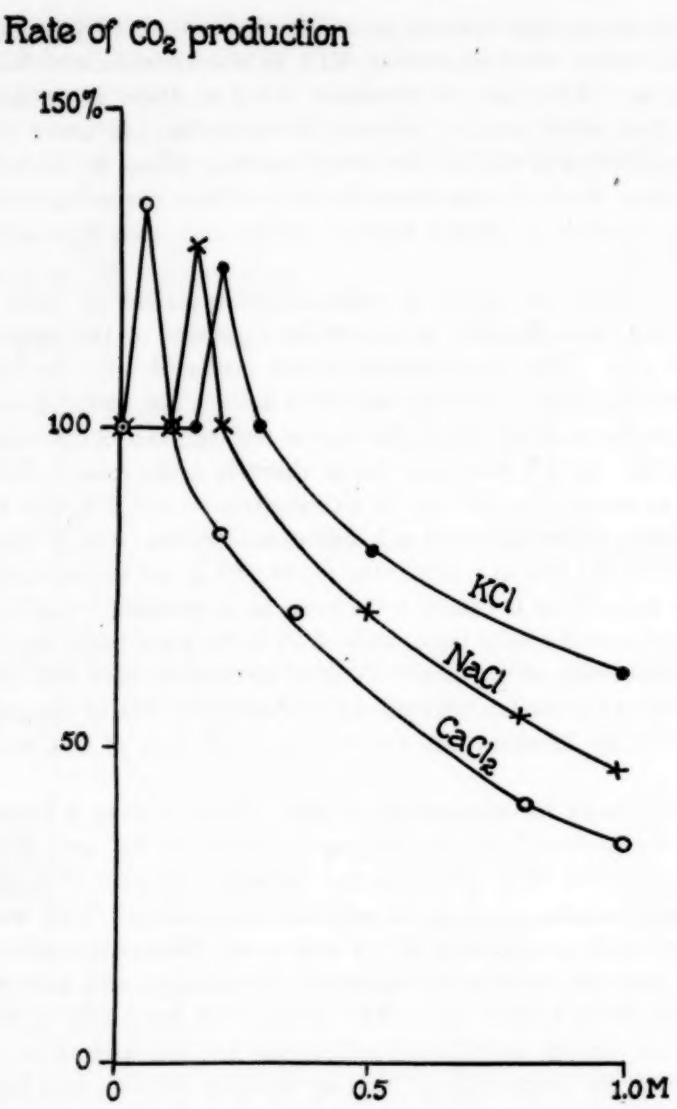

FIG. 4. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) as effected by salts. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Average of three experiments; probable error less than 3 per cent of the mean.

of these two salts (in the concentrations used) that produces normal respiration. It is of interest to note that Osterhout[6] and Lipman[1] also obtained two maxima with these salts in experiments upon wheat

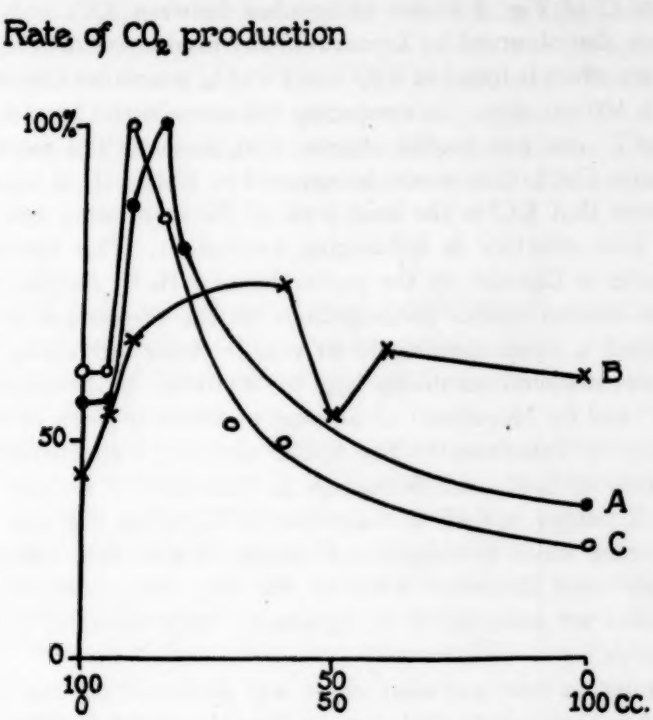

FIG. 5. Curves showing antagonism in the effect of salts on the respiration of *Bacillus subtilis*. Curve A, antagonism between NaCl, 0.8 M (left), and $CaCl_2$, 0.8 M (right); Curve B, antagonism between NaCl, 1 M (left), and KCl 1 M (right); Curve C, antagonism between KCl, 1 M (left), and $CaCl_2$, 1 M (right). The ordinates represent rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the salts used. Thus, in Curve A, the ordinate at the extreme left represents the rate in NaCl 0.8 M, while the ordinate at the extreme right represents the rate in $CaCl_2$ 0.8 M. The ordinate in the middle represents the rate in 50 parts NaCl 0.8 M + 50 parts $CaCl_2$ 0.8 M. The normal rate (which is taken as 100 per cent) represents a change in pH from 7.78 to 7.60 in about 30 seconds, varying according to the number of bacteria used. Curve A, average of two experiments; Curve B, average of five experiments; Curve C, average of three experiments. Probable error less than 3 per cent of the mean.

[6] Osterhout, W. J. V., *Bot. Gaz.*, 1909, xlviii, 98.

and upon the production of NH_3 by *Bacillus subtilis* respectively. The fact that both salts are monovalent may be a factor in explaining their peculiar behavior.

Curve C of Fig. 5 shows antagonism between KCl and $CaCl_2$. This was also observed by Lipman[1] in the production of NH_3. The maximum effect is found at 9 KCl to 1 $CaCl_2$, where the rate of respiration is 100 per cent. In comparing this curve with Curve A (NaCl and $CaCl_2$) one can readily observe that more KCl is required to antagonize $CaCl_2$ than would be required of NaCl. It is interesting to observe that KCl is the least toxic of the three salts, and that it is the least effective in influencing respiration. This agrees with the results of Lipman[1] on the production of NH_3 by *Bacillus subtilis*.

There are no similar investigations on the respiration of plants with which a comparison might be made. Some interesting studies have been made on sea urchin eggs by Warburg,[7] by Loeb and Wasteneys,[8] and by Meyerhof,[9] an account of which is given in a recent summary by Osterhout.[10] The results obtained with bacteria agree with those of Loeb and Wasteneys in that there is no rise in rate in NaCl, except in 0.15 M concentration in which the rise is only 30 per cent which is insignificant compared with that obtained by Warburg[7] and Meyerhof[9] (200 to 500 per cent). On the whole the results are more nearly in agreement with those of Loeb and Wasteneys.[8]

In order to find out what effect was produced on the bacteria while they were being acted upon by the salts, a few recovery experiments were tried. After the bacteria had remained in the salt solution for an hour, they were centrifugated and thoroughly washed in dextrose solution and centrifugated again. The supernatant fluid was then drained off, 2 cc. of dextrose solution were added, and their respiration was measured. It was found that within a period of less than $\frac{1}{2}$ hour the rate became normal.

A control experiment was made by substituting the same salt in the same molecular concentration as was removed from the bacteria

[7] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305; *Biochem. Z.*, 1910, xxix, 414.
[8] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1910, xxviii, 340; 1911, xxxi, 168.
[9] Meyerhof, O., *Biochem. Z.*, 1911, xxxiii, 291.
[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1919–20, ii, 1.

after centrifugating them to see whether or not the mechanical manipulation was responsible for the normal rate. The rate, however, showed no recovery under these conditions.

In order to find out whether the pH value of the liquid containing the bacteria changed when the salts were added, thereby influencing the rate of respiration, an indicator was added to this liquid in the apparatus and the pH value was observed to remain so nearly constant that the change in the rate of respiration could not be attributed to changes in alkalinity of the medium in which the bacteria were placed.

SUMMARY.

1. In relatively low concentrations of NaCl, KCl, and $CaCl_2$ the rate of respiration of *Bacillus subtilis* remains fairly constant for a period of several hours, while in the higher concentrations, there is a gradual decrease in the rate.

2. NaCl and KCl increase the rate of respiration of *Bacillus subtilis* somewhat at concentrations of 0.15 M and 0.2 M respectively; in sufficiently high concentrations they decrease the rate. $CaCl_2$ increases the rate of respiration of *Bacillus subtilis* at a concentration of 0.05 M and decreases the rate at somewhat higher concentrations.

3. The effects of salts upon respiration show a well marked antagonism between NaCl and $CaCl_2$, and between KCl and $CaCl_2$. The antagonism between NaCl and KCl is slight and the antagonism curve shows two maxima.

COMPARATIVE STUDIES ON RESPIRATION.

IX. The Effects of Antagonistic Salts on the Respiration of Aspergillus niger.

By F. G. GUSTAFSON.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, July 21, 1919.)

The relation of antagonistic salts to the respiration of higher fungi has received no attention. As the problems involved are of considerable interest the writer has made a beginning in this direction by conducting a series of experiments on *Aspergillus niger*.

It may be of interest to compare the results with those obtained on other organisms.

In experiments on sea urchin eggs Warburg[1] and Meyerhof[1] found that NaCl causes a rise in respiration which is inhibited by the addition of $CaCl_2$. Loeb and Wasteneys[1] found no such rise. The results with *Aspergillus* show a rise with lower concentrations of NaCl and a fall with higher concentrations. Since the highest rise in NaCl (24 per cent) is very small as compared with the rise of several hundred per cent obtained by Warburg and by Meyerhof, the results as a whole agree much more nearly with those of Loeb and Wasteneys. Still closer agreement is found in the results of Brooks,[2] who has used both sodium and calcium in studies on the respiration of bacteria. She finds an increase in the rate of respiration with certain concentrations of NaCl and $CaCl_2$ and a decrease in higher concentrations. A mixture of NaCl and $CaCl_2$ shows antagonism.

In some experiments on *Aspergillus niger* Kosinski[3] tested the effect of NaCl; although he seems to think that a 1.523 per cent (0.26M)

[1] For a summary of the experiments on sea urchin eggs see Osterhout, W. J. V., *J. Gen. Physiol.*, 1919–20, ii, 1.

[2] Brooks, M. M., *J. Gen. Physiol.*, 1919–20, ii, 5.

[3] Kosinski, I., *Jahrb. wiss. Bot.*, 1902, xxxvii, 154.

solution does not affect respiration, his data show an increase in respiration of 41 per cent.

The writer, in his experiments on *Aspergillus niger*, used NaCl and $CaCl_2$, as well as mixtures of the two salts. For measuring the production of CO_2 the apparatus described by Osterhout[4] was employed, in which the material is placed in one tube and the indicator in another.[5] This method is very accurate and simple. Care must be exercised to prevent any alkali from passing over from the tube containing it into the indicator tube, as this would vitiate the results.

By adding an indicator to the tube containing the fungus it can be shown that an acid is produced which is non-volatile (or practically so) since it does not disappear when a current of air (free from CO_2) is passed through the liquid for 15 or 20 minutes (under these conditions CO_2 would disappear in 5 minutes or less).

The apparatus is so constructed that a non-volatile acid cannot affect the color of the indicator which is being matched by the observer. The question may arise, however, whether the production of such an acid has any bearing on the interpretation of the results. If it is borne in mind that the problem is to ascertain the changes produced in the output of CO_2 under the influence of reagents it is evident that we need not consider the production of other acids except as intermediate stages or as by-products whose study is a problem by itself. This problem always exists, for wherever respiration goes on organic acids and other substances are produced. It seems best for the present to confine the investigation to the production of CO_2, leaving the study of other substances to the future.

The rate of production of CO_2 is obtained by taking the reciprocal of the time required to produce a definite change of color in the indicator tube.[6]

The fungus was grown and handled as previously described,[6] except that water distilled from a hard glass flask was used in place of tap water. The fungus, which forms a mat on the surface of the cul-

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17, 171.

[5] In this investigation five drops of a 0.01 per cent solution of phenolsulfonephthalein were added to 10 cc. of water. This solution was then used for comparison with the buffer tubes, which contained the same amount of indicator.

[6] Gustafson, F. G., *J. Gen. Physiol.*, 1918–19, i, 181.

tured solution, was rinsed in distilled water[7] to free it from any adhering nutrient solution, before putting it in the apparatus. The proper amount of material was then wound around the glass tube dipping into the solution in the respiration tube and secured in this position by tying it with a thread. This exposed the surface of the fungus to the liquid and also kept it from moving about and insured the smallest amount of mechanical injury. When the apparatus was in motion the air bubbles passing through the solution containing the organism carried the CO_2 given off by the fungus into the indicator tube, where the change in color was noted.

In all experiments pH 7.75 was the starting point and pH 7.42 the end-point. This gave the same range for every reading and a uniform change of 0.33 pH. The time required to produce this change under normal conditions varied from $2\frac{1}{4}$ to $3\frac{1}{2}$ minutes, depending upon the amount and condition of the material.

A number of preliminary experiments were performed without giving the fungus any nutrient while they lasted. When no nutrient was present the rate of respiration gradually fell below normal. For this reason it was thought best to add enough food to keep the control up to normal, so that any deviation from normal would be due to the action of the salt under investigation.

In starting an experiment the rate of respiration was first obtained in a 0.1 per cent solution of dextrose in distilled water. Unless the rate remained practically constant for at least 20 minutes in this solution, the material was rejected. The rate obtained in the dextrose solution is called the normal rate of respiration and in all calculations is taken as 100 per cent.

Several concentrations of dextrose were tried, but as all seemed to give the same results 0.1 per cent was chosen, as low viscosity is advantageous in the prevention of foaming. When the reagent used in the experiment was introduced the concentration of dextrose was thereby diluted one-half, so that during the experiment with the reagent it was only 0.05 per cent. In the numerous controls which were made the same thing was done, but no effect on the respiration was

[7] All water used in these experiments was distilled from a hard glass flask, and came in contact with hard glass only throughout the experiment.

noticed, and control experiments made in this way kept up to normal for several hours, or as long as the experiment lasted.

Various concentrations of NaCl were used. Lower concentrations such as 0.125M, 0.25M, and 0.5M caused a rise in respiration. At 1M the results were rather variable, some experiments showing a rise and others a fall. Solutions of 2M always gave a decided decrease which was followed by a small increase. The respiration then remained constant for more than an hour or to the end of the experiment. Figs. 1 and 2 give a graphic representation of the results obtained with various concentrations of NaCl.

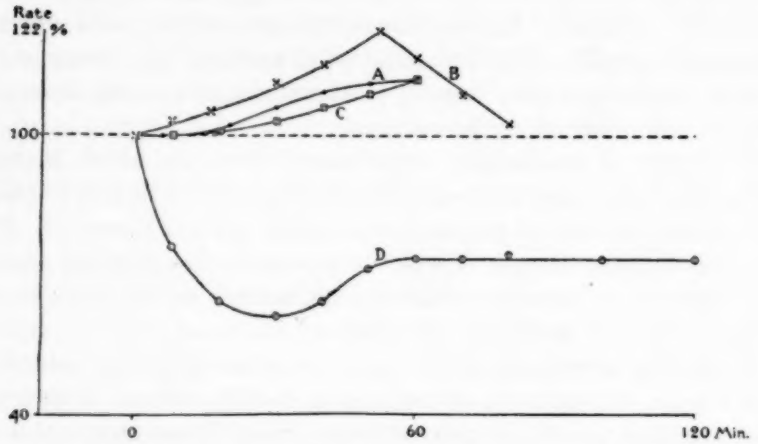

FIG. 1. Respiration of *Aspergillus niger*. The broken straight line to the left of the point marked 0 on the abscissa represents the normal rate of respiration before the addition of the salt. Curve A represents the respiration in 0.125M NaCl, Curve B respiration in 0.25M NaCl, Curve C respiration in 0.5M NaCl, Curve D respiration in 2M NaCl, and the broken line the control in 0.05 per cent dextrose. A, B, and C were in 0.05 per cent dextrose while D was in 1.5 per cent dextrose. Curves A, C, and D are each an average of 3, B of 4 experiments. Probable error less than 3 per cent of the mean.

The results with the lower concentrations are somewhat variable as to the amount of increase, as well as in respect to the time of maximum respiration. Thus in some experiments with 0.25M NaCl the maximum was reached at about 30 minutes after the introduction of the salt, while in most the maximum was not reached until at the end of 50 minutes.

This variability was of course to be expected with substances that are not more toxic than NaCl, where the least individual variation in the physiological activity of the cultures has a chance to exert its influence fully. This fact has already been noted in respect to some of the weaker anesthetics.[6] Though there are differences in the amount of increase and in the time when the maxima occur, yet there

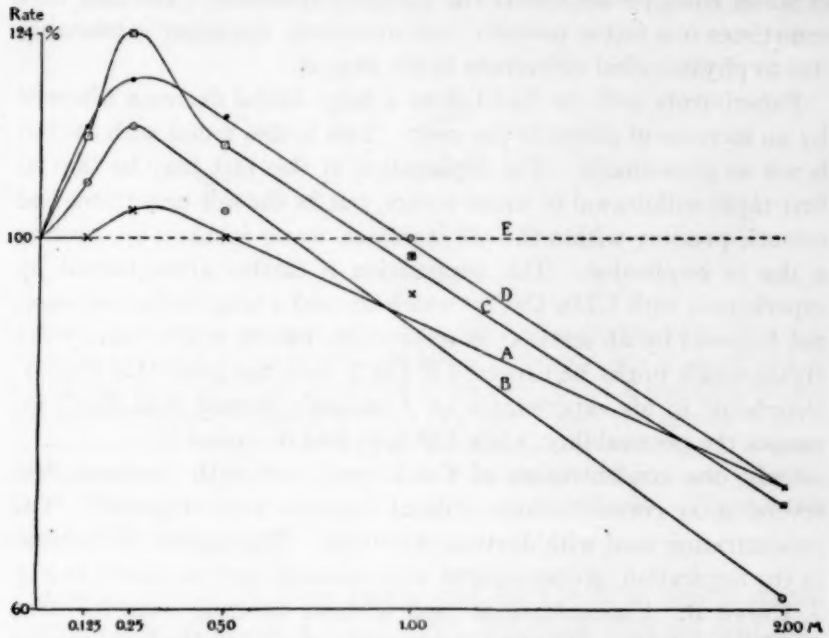

FIG. 2. Respiration of *Aspergillus niger*. The curves show rate plotted against concentration and represent the same experiments as shown in Fig. 1. Curve A represents the respiration at the end of 10 minutes exposure to NaCl, Curve B at end of 30, Curve C at end of 52, and Curve D at end of 60 minutes. Curve E represents the control in 0.05 per cent dextrose.

is no doubt about the general result. There is a distinct, though small, amount of increase in the rate of respiration, when *Aspergillus niger* is treated with NaCl at concentrations between 0.125 and 0.5M.

With 1M solutions of NaCl apparently contradictory results were obtained; *i.e.*, some experiments gave a large initial decrease followed by a rise to normal, others gave little or no initial decrease followed

by a rise above normal, while still other experiments gave only normal respiration.

The writer believes that these results are due to two factors acting on the fungus. One is the specific chemical action of NaCl which tends to stimulate, while the other, the osmotic pressure of the NaCl, tends to decrease the respiration. As is well known the abstraction of water from tissues lowers the rate of respiration. The fact that sometimes one factor prevails, and sometimes the other is probably due to physiological differences in the fungus.

Experiments with 2M NaCl show a large initial decrease followed by an increase of about 10 per cent. This is also found with 1M but is not so pronounced. The explanation of this fact may be that at first rapid withdrawal of water occurs, but as the salt penetrates and osmotic pressure within the cell increases, water is taken up, causing a rise in respiration. This supposition is further strengthened by experiments with 1.25M $CaCl_2$,[8] which showed a large initial decrease, not followed by an increase in respiration, but by a slow steady decrease which might be expected if $CaCl_2$ does not penetrate readily. Osterhout[9] in his experiments on *Laminaria* showed that NaCl increases the permeability, while $CaCl_2$ at first decreases it.

Only one concentration of $CaCl_2$ was used with dextrose, but several more concentrations without dextrose were employed. The concentration used with dextrose was 0.5M. This caused an increase in the respiration, giving a curve with rounded apex as shown in Fig. 3, Curve B. Concentrations used without dextrose were 0.3125M, 0.625M, and 1.25M. The first two caused a rise, while the last one only a decrease in respiration.

At this point it may be of interest to note that the effect of the salt seemed to be more pronounced when dextrose was absent than when it was present. This was especially the case with $CaCl_2$. Kosinski found that *Aspergillus niger* does not store up raw food material, and that as soon as it is taken out of a solution containing nutrient material it is in a starving condition.[10] He states that when this happens

[8] This concentration of $CaCl_2$ has approximately the same osmotic pressure as 2M NaCl.

[9] Osterhout, W. J. V., *Science*, 1911, xxxiv, 187; 1912, xxxv, 112.

[10] In the writer's experiments the rate of respiration began to decrease about 30 minutes after transfer from nutrient solution to distilled water.

plastic material is used in oxidation. If this is true it is easily conceivable that the oxidation of such material might not be affected in the same way by $CaCl_2$ as the oxidation of dextrose.

Experiments on antagonism between NaCl and $CaCl_2$ were also made. A solution containing 19 cc. of NaCl and 1cc. of $CaCl_2$ (both 0.5M) gave the best results. In this mixture the rate of respiration was practically normal (Fig. 3, Curve C). Other proportions gave more or less increase.

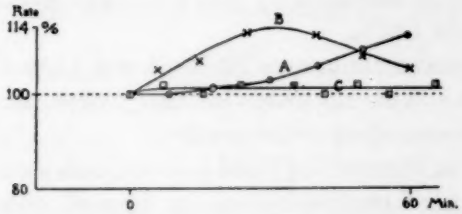

FIG. 3. Respiration of *Aspergillus niger*. The broken straight line to the left of the point marked 0 on the abscissa represents the normal rate of respiration in 0.1 per cent dextrose before the addition of the salt. Curve A represents the respiration in 0.5M NaCl, Curve B in 0.5M $CaCl_2$, and Curve C in a mixture of 19 cc. of NaCl and 1 cc. of $CaCl_2$ (both 0.5M). The control (dotted line) and C are practically identical. All these experiments were made in 0.05 per cent dextrose. Curve A is an average of 3, Curves B and C of 4 experiments. The probable error was less than 3 per cent of the mean.

In order to compare these results with the effects on growth spores were sown on solutions of NaCl and $CaCl_2$ (with 0.05 per cent dextrose), on various mixtures of these,[11] and on 0.05 per cent dextrose alone. For these experiments Petri dishes were used. The extent of germination was determined by microscopic examination.

The following results were noted: In NaCl there was no germination; in 49 cc. of NaCl+1 cc. of $CaCl_2$ some germination; 24 cc. of NaCl+ 1 cc. of $CaCl_2$, and 9 cc. of NaCl + 1 cc. of $CaCl_2$ showed a growth which increased in proportion to the amount of $CaCl_2$; 4 cc. of NaCl +1 cc. of $CaCl_2$ seemed to produce the best growth; 1 cc. of NaCl + 1 cc. of $CaCl_2$ and $CaCl_2$ alone showed fairly good growth; 0.05 per cent dextrose produced rather poor growth.

[11] Both NaCl and $CaCl_2$ were 0.5M and were dissolved in 0.05 per cent dextrose.

From these data it would seem that effects of $CaCl_2$ and NaCl on respiration are different from their effects on growth. This difference cannot be an osmotic effect, as the $CaCl_2$ solution, having a stronger osmotic pressure, would in that case be the one that would prevent growth to a greater extent, but this is not the fact.

SUMMARY.

1. In the presence of 0.05 per cent dextrose the respiration of *Aspergillus niger* is increased by NaCl in concentrations of 0.25 to 0.5M, and by 0.5M $CaCl_2$.

2. Stronger concentrations, as 2M NaCl and 1.25M $CaCl_2$, decrease the respiration. The decrease in the higher concentrations is probably an osmotic effect of these salts.

3. A mixture of 19 cc. of NaCl and 1 cc. of $CaCl_2$ (both 0.5M) showed antagonism, in that the respiration was normal, although each salt alone caused an increase.

4. Spores of *Aspergillus niger* did not germinate on 0.5M NaCl (plus 0.05 per cent dextrose) while they did on 0.5M $CaCl_2$ (plus 0.05 per cent dextrose) and on various mixtures of the two. This shows that a substance may have different effects on respiration from those which it has upon growth.

THE RELATIVE PHYSIOLOGICAL EFFECTS OF β- AND γ-RAYS UPON THE EGG OF NEREIS.

By ALFRED C. REDFIELD and ELIZABETH M. BRIGHT.

(From the Laboratory of Physiology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole.)

(Received for publication, June 17, 1919.)

As a preliminary to an investigation of the relative effects of β-rays of different velocities upon the egg of the marine worm, *Nereis limbata*, it is desirable to determine how far the reaction in question is due to the β- and to the γ-rays.[1] This information is also of importance in connection with a recently published[2] determination of the temperature coefficient of the action of the rays from radium upon these eggs since, as will be seen, it enables us to assign this coefficient to the action of the β-rays.

When unfertilized *Nereis* eggs are exposed to radiations from radium and then fertilized, the fertilization membrane which results is of unusual thickness.[3] We have shown that this reaction may be made to serve as a measure of radioactivity since a simple relation exists between the volume of the membrane formed by the eggs and the intensity of the radiation to which they have been exposed.[4] When used in this way the *Nereis* egg will give a measure of the intensity of the rays only in as far as they are physiologically effective. Our method has consisted in exposing a series of eggs for uniform periods of time to radiations passing through various thicknesses of aluminium and in determining the resulting membrane volumes. At the same time a series of eggs from the same female worm was exposed to various known intensities of radiation, produced by varying the distance between the eggs and the source of radiation, and evalu-

[1] Redfield, A. C., and Bright, E. M., *J. Gen. Physiol.*, 1919–20, ii, 31.
[2] Redfield, A. C., and Bright, E. M., *J. Gen. Physiol.*, 1918–19, i, 255.
[3] Packard, C., *J. Exp. Zool.*, 1915, xix, 323.
[4] Redfield, A. C., and Bright, E. M., *Am. J. Physiol.*, 1917–18, xlv, 374.

ated according to the formula of Wood and Prime.[5] A curve was then plotted relating the intensity of radiation to the membrane volumes resulting. This curve was used to determine the intensities of radiation reaching the first series of eggs through the various thicknesses of aluminium.

From data obtained in this way a coefficient could be obtained expressing the absorption of the physiologically effective radiation by aluminium. In our experiments the radioactive source has consisted of radium emanation, in equilibrium with its products, contained in a slender glass tube, the walls of which were sufficiently thick to absorb all the α-rays.[6] Such a preparation emits β- and γ-rays. The former are much less penetrating than the latter and possess as a result a much higher coefficient of absorption. If the physiological effect is due wholly or in large part to one of these types of radiation to the exclusion of the other, the absorption coefficient of the physiologically effective radiation should be of the same magnitude as the absorption coefficient of that type of radiation. If, on the other hand, both sorts of radiation contribute considerably to the physiological reaction, the absorption coefficient determined by it should have an intermediate value. Fig. 1 shows the results of three experiments. Against the thickness of the aluminium filters are plotted the logarithms of the per cent of the radiation passing through the filters, estimated from the effect upon *Nereis* eggs. Through these points we have drawn a line which corresponds to an absorption coefficient, μ, of 23.9 cm.$^{-1}$ estimated according to the equation

$$\frac{I}{I_o} = e^{-\mu d}$$

where I is the intensity of radiation passing through a thickness of aluminium d, and I_o is the intensity of the original beam.

The β-radiation from preparations such as we have employed is due chiefly to the disintegration of Radium *B* and *C*. The velocity

[5] Wood, F. C., and Prime, F., Jr., *Ann. Surg.*, 1915, lxii, 751.

[6] We are greatly indebted to Dr. William Duane and to Dr. R. B. Greenough, the Director of the Cancer Commission of Harvard University, for placing a supply of radium emanation at our disposal.

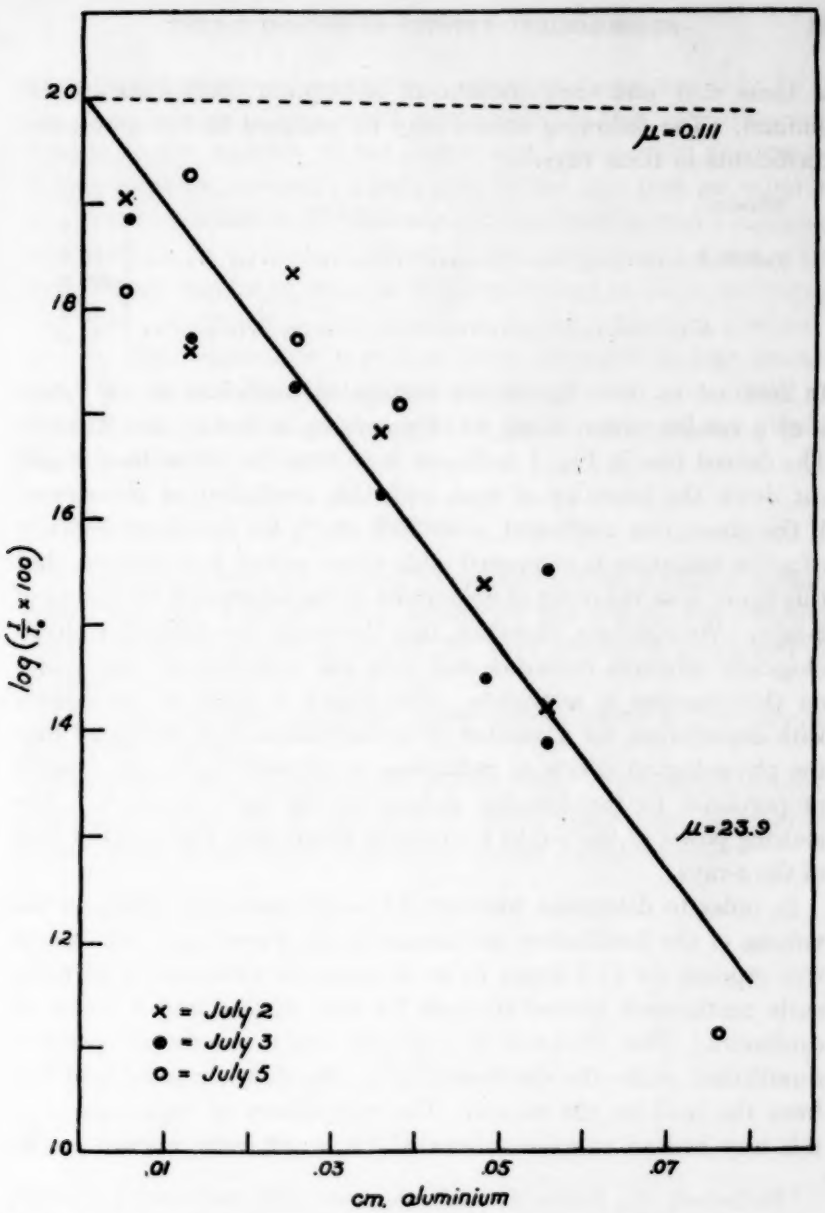

FIG. 1. Absorption of radiations from radium by aluminium measured by their effect upon the egg of *Nereis*. Thickness of aluminium measured in cm. along the abscissa. Per cent of transmitted radiation measured logarithmically along the ordinate. The dotted line indicates the absorption of γ-rays.

of these rays and their consequent absorption coefficients are not uniform. The following values may be assigned to the absorption coefficients of these rays.[7]

Substance.	μ $cm.^{-1}$
Radium *B*	13.1
	80.0
	890.0
" *C*	13.2
	53.0

In contrast to these figures the absorption coefficient of the γ-rays is of a smaller order, being 0.111 according to Soddy and Russell.[8] The dotted line in Fig. 1 indicates how little the filters used would cut down the intensity of rays with this coefficient of absorption. If the absorption coefficient, $\mu = 23.9 \cdot cm.^{-1}$, for the physiologically effective radiation is compared with these values it is obvious that this figure is of the order of magnitude of the absorption coefficient of β-rays. We conclude, therefore, that the β-rays constitute the physiologically effective radiation and that the influence of the γ-rays on this reaction is negligible. This result is quite in accordance with expectation, for a number of investigations have indicated that the physiological effects of radiations of different types are roughly proportional to the ionizing powers of the rays involved.[9] The ionizing power of the γ-rays is probably about only 1 per cent of that of the β-rays.

In order to determine whether the γ-rays have any effect on the volume of the fertilization membrane of the *Nereis* egg a lot of eggs was exposed for 11.5 hours to an intensity of radiation of 65 millicurie centimeters filtered through 1.9 mm. of lead and 1.7 mm. of cardboard. This thickness of lead completely absorbs the primary β-radiation while the cardboard stops the slow electrons sent out from the lead by the γ-rays. The membranes of eggs treated in this way had an average volume of 5.9×10^5 cubic microns, while

[7] Rutherford, E., Radioactive substances and their radiations, Cambridge, 1913, 225.

[8] Soddy, F., and Russell, A. S., *Phil. Mag.*, 1909, xviii, 620.

[9] Russ, S., *Arch. Middlesex Hospital*, 1912, xxvii, 16. Lazarus-Barlow, W. S., and Beckton, H., *ibid.*, 1913, xxx, 47.

the average volume of a control of unradiated eggs was only 1.5×10^5 cubic microns. This result would indicate that the γ-rays contribute to the reaction of the *Nereis* egg. How small this contribution must be, however, is indicated by the fact that an exposure of about 11 minutes to 65 millicurie centimeters of β- and γ-radiation will produce an equivalent change in the membrane volume. The γ-rays alone require an exposure 60 times as long as the β- and γ-rays together. Inasmuch as long exposures to low intensities produce a greater effect upon these eggs than short exposures to high intensities,[4] the intensity of the physiologically effective β-rays must be much more than 60 times as great as the intensity of the physiologically effective γ-rays.

SUMMARY.

When *Nereis* eggs are exposed to radiations from a tube of radium emanation, the walls of which absorb all the α-rays, the resulting physiological change is produced by rays having a coefficient of absorption of the order of 23.9 $cm.^{-1}$

This fact indicates that the physiological effect is due almost exclusively to β-rays.

The γ-rays alone can produce the reaction. To produce equivalent physiological effects exposure to γ-rays alone must be approximately 60 times as long as exposure to β- and γ-rays together.

THE RELATIVE PHYSIOLOGICAL EFFECTS OF β-RAYS OF DIFFERENT VELOCITIES.

By ALFRED C. REDFIELD and ELIZABETH M. BRIGHT.

(From the Laboratory of Physiology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole.)

(Received for publication, June 17, 1919.)

Insight into the nature of the action of any reagent upon protoplasm can be gained only through the correlation of its physical and chemical properties with its physiological effects. The present investigation is an attempt to discover whether any quantitative relation exists between the physiological effects of β-rays from radium of different velocities and their physical characteristics.

At the present time it is impossible to state definitely whether qualitative differences exist in the reactions of tissues to α-, β-, γ-, and x-rays[1] although the characteristic effects in accelerating and retarding cell division and growth appear to be the same.[2, 3] It is also indicated that the magnitude of the physiological action of these various kinds of rays corresponds roughly with their strength, as measured by their action in ionizing air.[4]

Several investigators have already undertaken to compare the effects of slow and fast β-rays from radium, but none of them have attempted to procure homogeneous groups of rays or to determine more than relatively the velocities of the rays used. Packard, who by means of a strong magnetic field and with the aid of mica filters

[1] For evidence of specific effects of different types of rays see Beckton, H., *Arch. Middlesex Hospital*, 1914–15, xxxii, 123. Chambers, H., and Russ, S., *ibid.*, 1912, xxvii, 29. Colwell, H. A., and Russ, S., Radium, x-rays and the living cell, London, 1915, 146.

[2] Lazarus-Barlow, W. S., and Beckton, H., *Arch. Middlesex Hospital*, 1913, xxx, 47. Packard, C., *J. Exp. Zool.*, 1916, xxi, 199. Richards, A., *Biol. Bull.*, 1914, xxvii, 67.

[3] Packard, C., *J. Exp. Zool.*, 1915, xix, 323.

[4] Russ, S., *Arch. Middlesex Hospital*, 1912, xxvii, 16. Lazarus-Barlow, W. S., and Beckton, H., *ibid.*, 1913, xxx, 47.

separated the β-rays, observed certain differences in their effects upon the eggs of *Arbacia* and *Nereis*, but in this investigation no attempt was made to compensate for the differences in intensity of the rays to which the cells were exposed.[3] Congdon compared the effects obtained by exposing seeds to a primary beam of β-rays with the effects of the same beam plus the soft rays, which were scattered back from the primary beam from a surface of lead, and concluded that in proportion to their energy content the slower rays have a greater retarding action than the more rapid rays.[5]

The development of a method of measuring the physiological action of radiations from radium has enabled us to examine the problem quantitatively. The method depends upon the fact first observed by Packard that the fertilization membranes of the eggs of the marine worm, *Nereis limbata*, are greatly enlarged if the eggs have been exposed to radium prior to fertilization.[3] We have shown that the extent of this change is a reliable measure of the intensity of radiation.[6] When used as instruments for measuring the strength of a beam of heterogeneous rays, the eggs will take account of each ray only in as far as it is effective physiologically. We have shown that the influence of the γ-rays upon these eggs is negligible when compared to the β-rays,[7] and have consequently neglected their consideration in interpreting our experimental results. The general plan of the investigation has been to expose *Nereis* eggs to approximately homogeneous beams of β-rays of various velocities, to compare the effects so produced with the effects produced on eggs from the same worm by a heterogeneous beam of β-rays of known intensity, and in this way obtain a measure of the relative *physiologically effective radiation* in each homogeneous beam. These values have then been compared with the relative strengths of each homogeneous beam as measured by their ability to ionize air. Before going into the details of the experiments it may be stated that they indicate that the ratio of the physiological effect and the ionizing power of β-rays is constant for all velocities.

[5] Congdon, E. D., *Arch. Entwcklngsmechn. Organ.*, 1912, xxxiv, 267.
[6] Redfield, A. C., and Bright, E. M., *Am. J. Physiol.*, 1917–18, xlv, 374.
[7] Redfield, A. C., and Bright, E. M., *J. Gen. Physiol.*, 1919–20, ii, 25.

Procedure.

Homogeneous groups of β-rays have been procured by the method commonly used by physicists, which depends on the fact that in traversing a magnetic field these negatively charged bodies are deflected in a direction normal to the field and as a consequence travel in curved paths. The relation between the velocity of a β-particle, u, the radius of curvature of its path, R, and the strength of the magnetic field, H, are given by the equation

$$HR = \frac{m\,u}{e}$$

where m is the mass of the particle and e its charge. For our purpose it will suffice to note that the relative velocity of the β-ray is measured by HR, the product of the radius of curvature and the strength of the magnetic field, which are predetermined experimental conditions. By making the usual assumptions concerning the value of e and of m for various velocities absolute values might be assigned to the velocities of the beams of β-rays which we have used.

The apparatus which we have employed is illustrated in Fig. 1. A solenoid, 50 cm. long, was wound about a hollow water jacket, WJ. Knowing the number of turns of wire in the solenoid, its length, radius, and the strength of current passing through it, the strength of the magnetic field, H, in its center could be calculated directly. A stream of water circulating through the water jacket prevented the heat generated in the solenoid from increasing the temperature of the space within. Into this space could be introduced an arrangement for exposing the eggs to rays separated into homogeneous groups by the magnetic field. This device consisted of two lead blocks so arranged that a slender glass tube containing radium emanation, E, was supported 1 cm. from a slit, S, through which a beam of rays escaped.[8] The width of the slit was 0.1 cm. in some experiments, 0.065 cm. in others. When undeflected, the rays leav-

[8] We are greatly indebted to Dr. William Duane for indispensable advice in designing our apparatus, and to Dr. R. B. Greenough, the Director of the Cancer Commission of Harvard University, for placing a supply of radium emanation at our disposal.

ing this slit passed on through a distance of 2 cm. and impinged at *A* upon a glass plate on which the eggs were spread. In the presence of a magnetic field a slow ray, travelling in a course having a small radius of curvature, would strike the glass plate at *C* while a fast

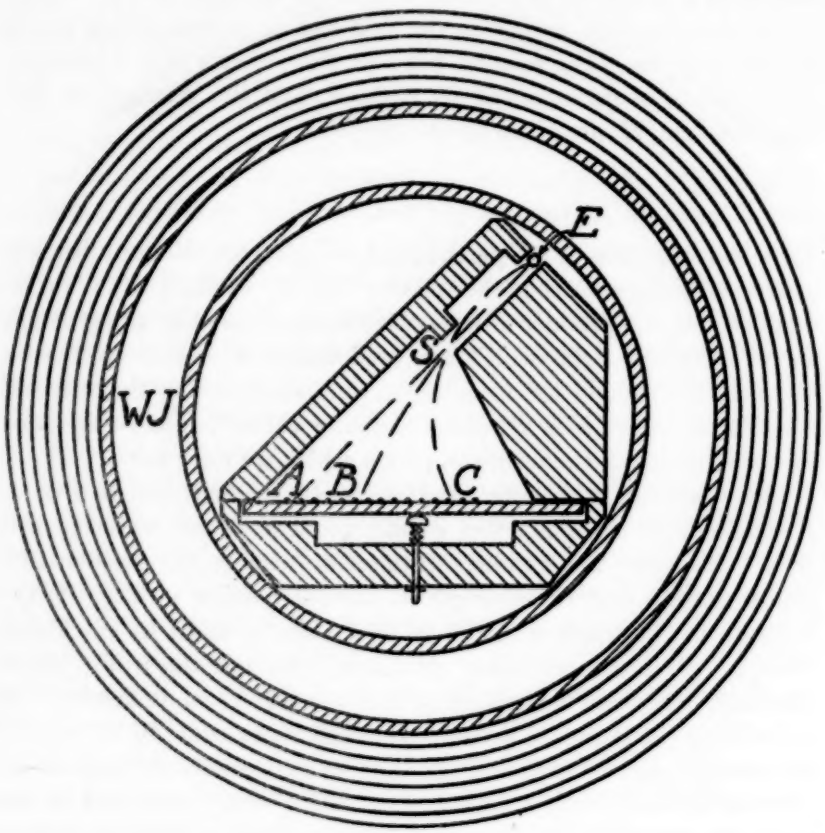

FIG. 1. Diagram of apparatus for exposing *Nereis* eggs to homogeneous groups of β-rays of different velocities.

ray travelling more nearly in a straight line would strike at *B* nearer to *A*. Fig. 2 is a photographic record of the course of the direct and deflected beam.

Two procedures were employed to expose eggs to beams of rays of different velocities. In one the magnetic field, *H*, was kept con-

stant while the radius of curvature, R, was varied by placing the eggs at different positions on the glass plate. In the other the magnetic field was varied by altering the current flowing through the solenoid, while the radius of curvature was kept constant by placing each lot of eggs in the same position on the glass plate.

In the former procedure eggs were spread uniformly over the plate in a thin film of sea water and radiated for 1 or 2 hours in the presence of a strong magnetic field.[9] Slender filaments of glass cemented across the plate divided it into chambers, each at a measured distance from A, and prevented the eggs from moving from the position which

FIG. 2. Photograph of the course of the β-rays when undeflected, A, and deflected, B, by a magnetic field of about 450 gauss. The upper picture was made by placing the photographic plate against the end of the lead blocks which define the beam, so that it was struck a glancing blow by the rays. The lower picture was made by placing the photographic plate under the lead blocks in the position occupied by the glass plate which supported the eggs.

they occupied while being radiated. At the completion of the period of radiation the plate was removed from the apparatus and the eggs were fertilized by blowing a shower of sperm suspended in sea water upon them with an atomizer. This procedure was essential in order to prevent eggs being washed from one compartment on the plate to another. About 40 minutes after fertilization, a cover-glass was laid across the glass filaments, which protected the eggs from being crushed, and measurements of the thickness of the membranes of

[9] Preliminary experiments established the fact that exposure to the magnetic field alone had no effect upon the membrane of the *Nereis* eggs.

the eggs were begun. In making these measurements one egg from each compartment was measured in turn, then a second from each compartment, then a third, and so on until ten or twelve eggs from each compartment had been measured, by which time the eggs had begun to divide and it was impossible to obtain further exact measurements. In this way we were assured that the figures for each compartment were made the same average time after fertilization, a matter of much importance. This procedure had the advantage that all the eggs were radiated at one time after they had been freshly removed from the female worm. Its disadvantage lay in the fact that it was impossible to measure very many eggs from each lot since all the lots had to be measured at once.

The alternative procedure consisted in placing successive lots of eggs in the same position on the glass plate so that each lot was exposed to rays travelling through the same path. In this case R, the radius of curvature of the effective beam, remained constant. Each lot of eggs was exposed for an equal period of time, 1 or 2 hours, but with a different amount of current flowing through the solenoid, so that the strength of the magnetic field, H, varied. At the termination of the period of radiation each lot of eggs was washed into a watch-glass of sea water, fertilized, and after a uniform period the membranes of twenty-five eggs were measured. This procedure possessed the advantage that a larger number of eggs from each lot could be measured, but it was so time-consuming that only a limited number of homogeneous beams could be measured in each experiment. Moreover, as the experiment proceeded measurements were necessarily made with eggs which had been removed from the female worm for longer and longer periods. We have some reason to suspect that these eggs become slightly less sensitive to radiation after they have stood in sea water for a long period.

The figures obtained from such experiments as these give a measure of the amount of physiological effect from each beam of homogeneous radiation. We have shown, however, that the amount of physiological effect, as indicated by the volume of the fertilization membrane of *Nereis* eggs, is not a direct measure of the intensity to which they have been exposed. What we desire is a measure of the physiologically effective radiation in each group of homogeneous rays which

can be compared with a measure of the same beam obtained by some standard physical method. Since we have shown that the volume of the membrane is a direct function of the logarithm of the intensity of radiation through a considerable range of membrane volumes, it is a simple matter to calibrate each lot of eggs so that the intensity of radiation can be approximated from any known membrane volume.[6] To do this a series of measurements was made of the physiological effects of various intensities of the entire radiation from a tube of radium emanation, the intensity being determined by the distances between the tube and the eggs according to the formula of Wood and Prime.[10] From data obtained in this way a curve, such as that shown in Fig. 3, was drawn relating the intensity of radiation with the resulting membrane volume. This curve was then used to determine the relative intensity of physiologically effective radiation in each beam of homogeneous β-rays by finding on the curve the intensity which corresponds to the average volume of the membranes of each lot of eggs.

Having obtained in the preceding manner a measure of the relative intensity of the physiologically effective radiation in each beam of β-rays of homogeneous velocity it became necessary to determine the total intensity of each beam by some standard physical method. Although the relative number of β-particles of various velocities given off by tubes of radium emanation in equilibrium with its products has been determined with precision,[11, 12] two considerations made it desirable to obtain this information with the apparatus used for the physiological determinations. In the first place the slit, S, used to separate the rays into homogeneous beams was of finite dimensions so that it was only possible to obtain an approximation to homogeneity. Since our physiological method of measurement was relatively insensitive it was necessary to keep the distances through which the rays travelled short and the slit wide in order that the effect upon the eggs might be great enough to be measured accurately. Another source of error in the method was due to the fact that a considerable

[10] Wood, F. C., and Prime, F., Jr., *Ann. Surg.*, 1915, lxii, 751.
[11] Wilson, W., *Proc. Roy. Soc. London, Series A*, 1909, lxxxii, 612.
[12] Wilson, W., *Proc. Roy. Soc. London, Series A*, 1911, lxxxv, 240.

number of rays struck the edges of the slit and were "reflected" in all directions so that all the beams were contaminated by a considerable number of rays of velocity considerably greater or smaller than the modal velocity of the beam. The only way the contribution of these scattered rays to each beam could be accounted for was a direct physical measurement made with the same apparatus as was used for the physiological experiments.

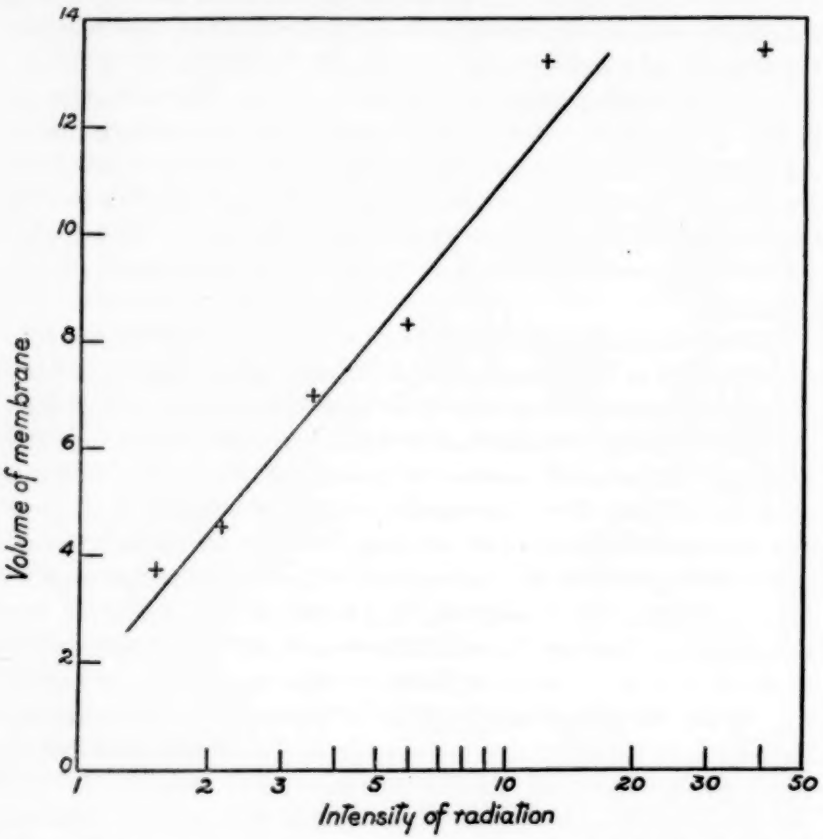

FIG. 3. Curve used to determine the relative intensity of the physiologically effective radiation from its effect on the membrane of *Nereis* eggs. The points are experimental results obtained with known intensities of heterogeneous radiation. The line is the curve used in calibrating the experimental results with homogeneous beams. Intensities measured in millicurie centimeters along the abscissa. Membrane volumes measured in 100,000 cubic microns along the ordinate. Experiment of August 29, 1918.

In order to calibrate the "spectrum" of β-rays the apparatus was modified as shown in Fig. 4. To make more room the water jacket was removed from the interior of the solenoid, and a brass tube arranged to extend into the center of the coil and support a small ioni-

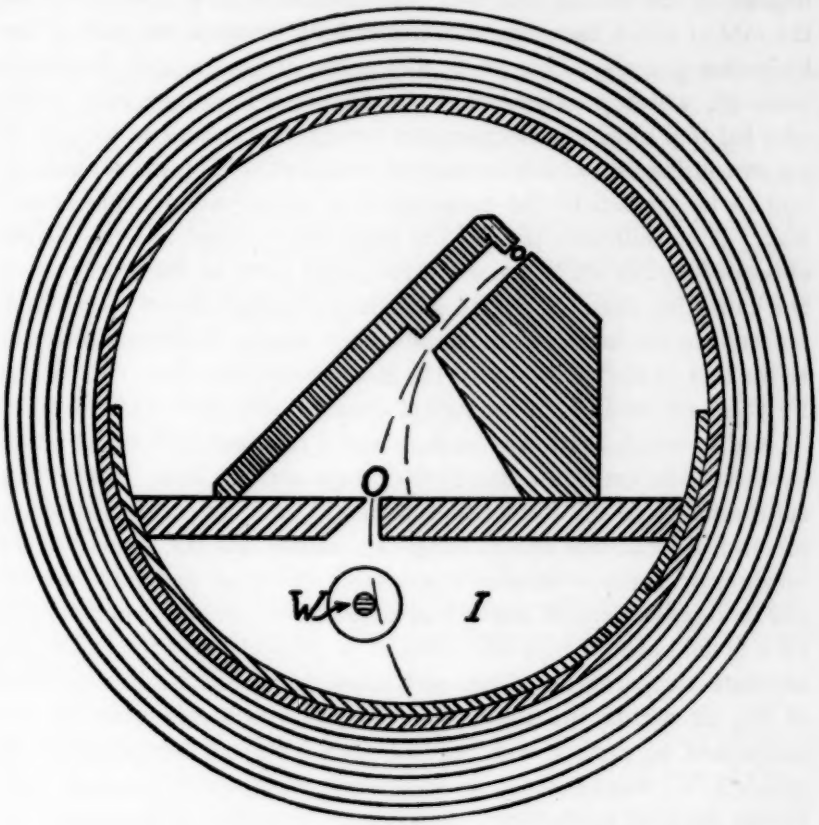

FIG. 4. Diagram of arrangement used to determine the relative ionizing powers of homogeneous groups of β-rays to different velocities.

zation chamber, *I*. The roof of this chamber consisted of a lead plate 3 mm. thick which prevented any β-rays from entering the chamber except through a hole, *O*, 1 mm. wide and 1 cm. long. The lead blocks which supported the tube of radium emanation rested

upon the roof of the ionization chamber, and could be slid back and forth so that a beam of β-rays of any desired radius of curvature entered the hole. When these rays passed through the ionization chamber the air in it became ionized. The relative number of ions formed by the various beams of homogeneous rays was measured by the rate at which they conducted electricity between the wall of the ionization chamber, charged to 120 volts, and a suitably insulated wire, *W*, which projected into the chamber from one end. This wire led to a quadrant electrometer arranged as described by Duane for measuring ionization currents of this sort.[13] Several corrections had to be applied to the measurements of ionization made in this way. A considerable part of the ionization current was due to the effects of γ-rays which passed through all parts of the lead roof of the ionization chamber and to secondary β-radiations set up by them on striking the walls of the chamber. A smaller increment was due to the leak of electricity across the insulation of the wire. To correct for these the roof of the ionization chamber was replaced by a piece of lead in which there was no hole and a measurement made which expressed the ionization due to the γ-rays and the leak. This value subtracted from the former measurement, gave an expression of the amount of ionization due to the β-rays alone. As the air within the ionization chamber became warm from the heat generated in the coil its density became less and as a result the number of ions formed by a given beam of rays was reduced in proportion to the rise in the absolute temperature. Care was taken to record the temperature of the air within the solenoid at the time each measurement was taken and to correct each reading to a standard temperature, for which 27°C. was chosen. In addition a slight correction was made for the decay of the radium emanation in the course of the experiment. By these procedures figures were obtained expressing in arbitrary units the relative ionization produced by each of the beams of β-rays which had been measured physiologically, and under exactly the same conditions.

[13] Duane, W., *Boston Med. and Surg. J.*, 1917, clxxvii, 787.

Results.

Seventeen experiments were conducted on *Nereis* eggs with these methods. Of these nine of a preliminary nature were rejected either because insufficient data were obtained for determining the effective

TABLE I.

Nereis Eggs Exposed to Homogeneous Beams of β-Rays Separated by a Uniform Magnetic Field but Travelling through Paths of Various Radii of Curvature.

July 9, 1918. H = 477 gauss. R variable. 0.1 cm. slit. Strength of emanation = 46 millicuries. Time of radiation = 90 min. Twelve eggs measured in each lot.

HR	Volume of membrane.	Relative effective radiation.	Relative ionization.	Ionization. / Effective radiation.
gauss cm.	10^5 μ^3			
840	4.40	19.8	20.0	1.01
965	4.50	23.5	23.0	0.98
1,120	6.10	28.1	44.0	1.57
1,315	7.90	49.1	62.0	1.26
1,595	11.60	82.5	80.0	0.97
1,990	12.55	98.5	96.5	0.98
2,600	12.65	100.0	100.0	1.00
3,445	11.60	82.5	95.5	1.16
5,010	10.90	73.6	78.0	1.06

Aug. 21, 1918. H = 502 gauss. R variable. 0.065 cm. slit. Strength of emanation = 58.4 millicuries. Time of radiation = 120 min. Eight to twelve eggs measured in each lot.

932	6.00	48.0	35.4	0.74
1,070	6.15	50.0	46.1	0.92
1,235	6.35	51.7	62.2	1.20
1,475	6.50	54.7	80.8	1.48
1,805	7.30	67.4	97.3	1.44
2,250	8.65	100.0	100.0	1.00
3,015	8.05	81.6	91.5	1.12
4,230	7.90	77.5	72.5	0.94
6,560	5.80	45.0	47.8	1.06

intensity from the physiological change obtained, or because too few determinations were made, or because the change in the membrane volumes obtained were too small for accurate measurement. One experiment was rejected because the measurements obtained were

TABLE II.

Nereis Eggs Exposed to Homogeneous Beams of β-Rays Separated by a Variable Magnetic Field but Travelling through Paths of the Same Radius of Curvature.

Aug. 1, 1918. H variable. R = 8.45 cm. 0.065 cm. slit. Strength of emanation = 51.5 millicuries. Time of radiation = 60 min. Twenty-five eggs measured in each lot.

HR	Volume of membrane.	Relative effective radiation.	Relative ionization.	$\frac{\text{Ionization.}}{\text{Effective radiation.}}$
gauss cm.	10^6 μ^3			
0	3.95	49.0	53.5	1.09
1,070	5.61	89.5	85.0	0.95
1,510	5.71	92.0	96.0	1.04
2,265	6.30	100.0	97.5	0.98
3,060	5.71	92.0	87.0	0.95
4,220	5.50	84.5	68.0	0.81

Aug. 29, 1918. H variable. R = 13.1 cm. 0.065 cm. slit. Strength of emanation = 70 millicuries. Time of radiation = 90 min.

0	3.70	55.0	76.6	1.39
1,560	6.05	100.0	100.0	1.00
2,860	5.80	93.5	96.5	1.03
4,100	5.70	91.5	82.8	0.91
5,340	4.10	62.5	66.7	1.07
6,570	4.85	75.5	57.5	0.76
7,320	3.35	51.0	52.5	1.03

Aug. 29, 1918. H variable. R = 8.45 cm. 0.065 cm. slit. Strength of emanation = 70 millicuries. Time of radiation = 90 min.

0	2.90	46.0	53.5	1.16
1,010	5.20	81.0	83.6	1.03
1,845	5.80	92.0	100.0	1.09
2,645	6.10	100.0	93.0	0.93
3,440	4.75	72.0	78.6	1.09
4,240	5.60	88.5	68.2	0.77
5,040	4.00	60.0	55.0	0.92

Aug. 30, 1918. H variable. R = 7.65 cm. 0.01 cm. slit. Strength of emanation = 60 millicuries. Time of radiation = 75 min.

0	4.35	38.2	35.2	0.92
531	5.55	53.6	48.0	0.90
1,305	5.60	54.0	72.0	1.33
2,045	8.50	88.6	95.0	1.07
2,765	9.80	105.0	100.0	0.95
3,485	9.10	94.8	98.7	1.04
4,210	9.30	99.0	90.2	0.91
4,925	6.60	64.0	81.5	1.27

TABLE II—*Concluded.*

Aug. 30, 1918. H variable. R = 5.45 cm. 0.01 cm. slit. Strength of emanation = 60 millicuries. Time of radiation = 75 min.

HR	Volume of membrane.	Relative effective radiation.	Relative ionization.	Ionization. / Effective radiation.
gauss cm.	$10^6\ \mu^3$			
0	3.65	30.2	32.5	1.08
392	4.25	40.7	38.3	0.94
935	4.30	42.0	59.0	1.40
1,405	6.45	70.0	77.1	1.10
2,000	8.70	101.0	92.5	0.92
2,490	8.55	100.0	100.0	1.00
3,000	9.00	104.5	94.5	0.91
3,520	6.70	72.2	91.5	1.27

so irregular that it is our belief that their inclusion would only serve to obscure the truth. Seven experiments remained which we consider satisfactory in every respect and their results are presented in Tables I, II, and III, and Figs. 5 and 6. It may be added that the rejected experiments, as far as can be judged, agree with the data here presented in showing maximum and minimum effects for beams of β-rays of corresponding velocities.

Table I contains the results of two experiments made with the procedure in which the magnetic field, H, remained constant while the radius of curvature, R, of the effective beam of β-rays varied with each lot of eggs. One of these experiments is illustrated graphically in Fig. 5. Table II contains the results of five experiments made with the procedure in which the strength of the magnetic field, H, varied, while the radius of curvature, R, in which the effective beam of β-rays moved was the same for each lot of eggs. One of these experiments is illustrated graphically in Fig. 6. Examination of the tables will show that the intensity of the radiation in each beam as measured physiologically, indicated in the third column of the table, and the amount of ionization produced by each beam in air, indicated in the fourth column, both increase rapidly up to a maximum at about the same value of HR and then fall off again as HR increases further. In the fifth column of these tables the ratio of the relative amount of ionization and the relative physiologically effective radiation is

given. The units in which these values are expressed are arbitrarily chosen so that if a direct relation exists between the ability of β-rays of any velocity to act upon living cells and to ionize air the figures in this column will approach a constant with a value about 1.0. This is seen to be the case. Individual figures fluctuate considerably from this value, as is to be expected in constants derived from physio-

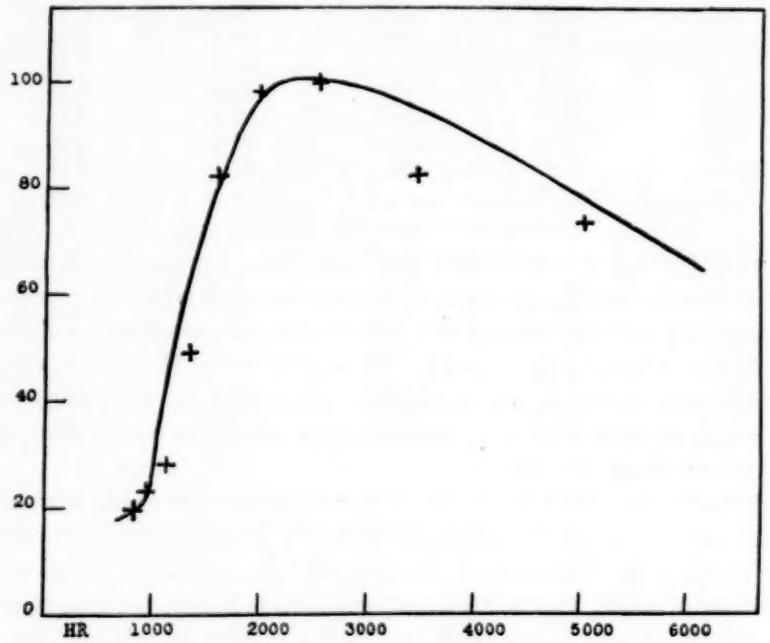

FIG. 5. The solid line represents the relative ionization, measured along the ordinate, produced by β-rays of different velocities, measured along the abscissa. The points represent the relative physiologically effective radiation in the same beams, as determined with *Nereis* eggs. For data see Table I, experiment of July 9, 1918. H = 477 gauss. R variable.

logical data, but the deviations do not show any correlation with the velocity of the β-rays under consideration. We have collected all the constants from Tables I and II into groups according to the velocity of the β-rays and have presented the average for each group in Table III. When this is done the fluctuations in the individual constants balance out and it becomes clearly apparent that *as the velocity of*

the rays increases the ratio between their physiological effect and their ionizing power remains constant.

It has been shown by Wilson that a number of slow β-rays produce many more ions per cm. path through air than the same number of fast ones.[12] Since we have shown that there is a constant relation

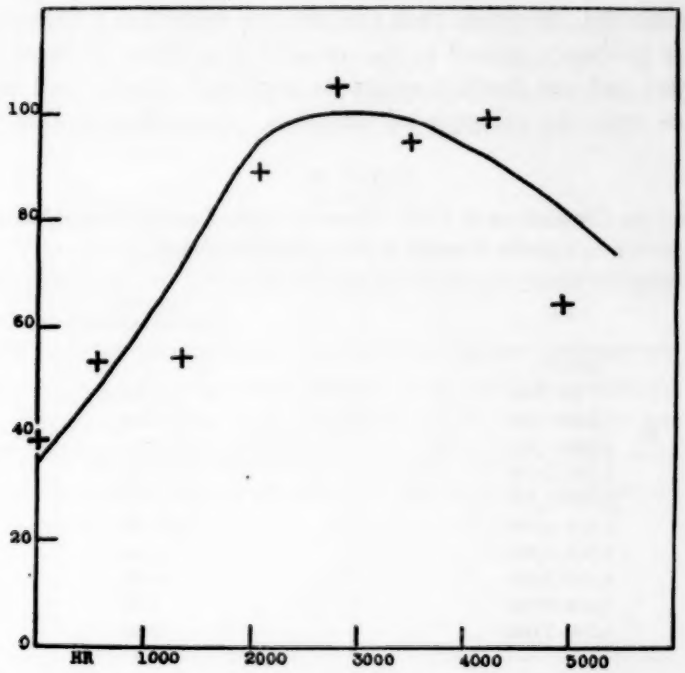

FIG. 6. The solid line represents the relative ionization, measured along the ordinate, produced by β-rays of different velocities, measured along the abscissa. The points represent the relative physiologically effective radiation in the same beams, as determined with *Nereis* eggs. For data see Table II, experiment of August 30, 1918. H variable. R = 7.65 cm.

between the ionization produced in passing through the depth of the ionization chamber and the physiological effect produced in passing through the *Nereis* eggs by a β-ray of any velocity, it may be concluded that in passing through a cell a number of slow β-rays will produce much more physiological effect than the same number of fast β-rays.

The finding that the physiological action of the β-rays is closely related to its ionizing power is compatible with the view which has been vaguely suggested by many investigators and definitely formulated by Joly[14] and Richards and Woodward[15] that the physiological effects of x-rays and radiations from radium are due primarily to their ionizing effects upon substances in the protoplasm. It must be remembered, however, that the relative ionization produced by β-rays is probably related to the relative absorption of these rays by matter and our findings might be explained equally well in accordance with the principle of Grotthus. According to this view

TABLE III.

Averages of the Constants in the Fifth Column of Tables I and II Grouped according to the Velocity of the β-Rays Employed.

HR	$\frac{\text{Ionization.}}{\text{Effective radiation.}}$
gauss cm.	
0– 500	1.13
500–1,000	0.99
1,000–1,500	1.20
1,500–2,000	1.06
2,000–2,500	1.01
2,500–3,000	0.99
3,000–4,000	1.10
4,000–5,000	0.92
5,000–6,000	1.02
6,000–7,000	0.95

slow β-rays produce more physiological change than a corresponding number of fast β-rays because they are absorbed to a correspondingly greater degree. Ionization of some of the constituents of protoplasm need not necessarily be involved in the transformation of the energy consumed in producing the physiological change. To establish the fact that ionization of the cell constituents actually occurs under the influence of these rays, quantitative data of another sort must be forthcoming.

[14] Joly, J., *Proc. Roy. Soc. London, Series B*, 1914–15, lxxxviii, 262.
[15] Richards, A., and Woodward, A. E., *Am. J. Roent.*, 1917, iv, 564.

The conclusion of Congdon will be seen to be at variance with our experimental result.[5] In his experiments it was observed that the addition of enough soft β-rays to a heterogeneous beam to increase its ionizing power 25 per cent caused an average retardation of 35 per cent in the rate of growth of seeds exposed to the radiations. It is to be regretted that Congdon did not determine how greatly the retardation would have been increased by strengthening the primary beam by 25 per cent without changing its character. If this procedure had yielded retardation of less than 35 per cent we could then feel sure that the discrepancy in our results was a real one.

SUMMARY.

1. The physiological effect upon the eggs of *Nereis* of homogeneous groups of β-rays of different velocities is proportional to their ability to ionize air.

2. β-rays of low velocity produce a greater amount of physiological change than the same number of rays of high velocity.

3. These conclusions are consistent with, but do not prove, the view that the physiological effects of radiations from radium and x-rays are due to the production by them of an ionization of some substance in the egg.

CHANGES IN PROTOPLASMIC CONSISTENCY AND THEIR RELATION TO CELL DIVISION.

By ROBERT CHAMBERS.

(From Cornell University Medical College, New York City, and the Marine Biological Laboratory, Woods Hole.)

(Received for publication, July 29, 1919.)

I. Periodic Changes in Consistency of the Egg Cytoplasm after Fertilization and during Cleavage.

On fertilization an increase in the viscosity of the semifluid cytoplasm of the sea urchin egg was noticed by Albrecht[1] and recently fully demonstrated by Heilbrunn.[2] Heilbrunn based his conclusions on his observation that a greater centrifugal force is necessary to stratify the cell constituents of an egg after fertilization than before. I[3] have presented evidence, from microdissection studies on the sand-dollar egg and the egg of *Cerebratulus*, that the increase in viscosity is associated with the appearance and growth of the aster.

Upon entrance of the spermatozoon into the egg a diminutive aster makes its appearance as a ball of a jelly-like consistency in the immediate vicinity of the sperm head. This aster, with the sperm nucleus, moves inward as it steadily increases in size until, when its center comes to lie in or near the center of the egg, its radiations extend throughout the whole egg. During this migration the sperm nucleus comes into contact with the egg nucleus. The aster then develops completely around the two nuclei, which fuse to constitute the cleavage nucleus.

The development of the sperm aster in the sea urchin egg is at its height within 10 to 15 minutes after fertilization. This is the

[1] Albrecht, E., Untersuchungen zur Struktur des Seeigeleies, *Sitz-ber. Ges. Morph. u. Physiol.*, 1898, xiv, 133.

[2] Heilbrunn, L. V., Studies in artificial parthenogenesis. II. Physical changes in the egg of *Arbacia*, *Biol. Bull.*, 1915, xxix, 149.

[3] Chambers, R., Jr., Microdissection studies II. The cell aster: A reversible gelation phenomenon. *J. Exp. Zool.*, 1917, xxiii, 483.

time when Heilbrunn informs me he found the egg substance to be of maximum viscosity.

The increase in viscosity of the egg cytoplasm is produced by an influence spreading out in all directions from the center of the aster. While this occurs the central hyaline area of the aster (the hyaloplasmsphere of Wilson) increases in size, and there is strong evidence[3] that this is due to the accumulation of a hyaline liquid which separates out of the semisolidifying cytoplasm and flows in very fine converging streams to the center of the aster. It is possible that this and kindred phenomena give to the aster the appearance of radiations from a common center. The consistency of the cytoplasm incorporated in the aster diminishes in firmness on passing from the interior of the aster to its exterior, being greatest in the region bordering on the centrosphere and least at the periphery.

The disappearance of the sperm aster, in the opinion of the writer, occurs through a process of liquefaction. During the liquefaction the substance of the centrosphere collects into two areas at opposite poles of the cleavage nucleus. The experiments to be described in this paper indicate that shortly before cleavage each of these areas becomes a center around which the cytoplasm commences again to pass into a semisolid state. The radial configuration about these areas constitutes the amphiaster. The comparatively firm consistency that the egg now attains for the second time since fertilization is due to two masses, the two asters, instead of to a single aster as was the case shortly after the entrance of the sperm. The importance of this phenomenon in its bearing on cell division is discussed in the last part of this paper.

Experiment 1.—The consistency exhibited by the protoplasm of the sea urchin egg at various periods from the moment of fertilization until the completion of the first cleavage, was ascertained by careful probing with the microdissection needle.

Immediately after fertilization the cytoplasmic granules readily flow by the moving needle. After the sperm has entered the egg, the sperm aster constitutes a comparatively firm mass which gradually increases in size as it moves to a central position in the egg. When the sperm aster is at its full development the highly viscous state of the cytoplasm is detected by the needle. Illustrations of this

are given in a former paper.[3] The cytoplasmic granules, instead of being readily dislocated by the moving needle, are held as in a jelly, and movements of the needle produce torsions of the entire egg substance. This condition is at its height 10 to 15 minutes after fertilization.

15 to 20 minutes after fertilization, the radiations of the aster begin to fade from view, with a reversal in the cytoplasm of the semi-solid to a more fluid state. The cytoplasmic granules are now easily dislocated by the moving needle. The more prominent radiations disappear first, while the finer ones persist for some time, owing probably to the viscid nature which the cytoplasm always maintains. The liquid substance of the central hyaline area now flows over the nucleus to its two poles, beyond which it often extends. This causes the appearance characteristic of this stage, of a hyaline streak plainly visible in the otherwise granular cytoplasm of the egg. Toward the end of this stage, which lasts for about 20 to 30 minutes, the hyaline substance finally collects into two semispherical masses lying at the two poles of the nucleus.

Shortly before cleavage, about 40 to 50 minutes after fertilization, an increase in firmness sets in, spreading radially from each of the two centers situated at the poles of the nuclear spindle. This constitutes the amphiaster. The egg elongates, the long axis passing through the two centers of the amphiaster. The cleavage furrow now appears and the egg rapidly divides. The time of appearance of the amphiaster until completion of cleavage lasts from 10 to 15 minutes. The increased viscosity of the egg during this amphiaster stage could be more easily demonstrated by the needle in the eggs of *Echinarachnius* and *Cerebratulus* than in those of *Arbacia*.

After completion of the cleavage process, there are indications that the firmness of the cytoplasm persists in the two blastomeres while they are still more or less spherical. Within 10 to 15 minutes after cleavage the two blastomeres crowd up against one another, each assuming a more nearly hemispherical shape. At this stage their cytoplasm is again quite fluid.

These observations demonstrate a pronounced periodicity in the physical state of the egg subsequent to fertilization and during the first cleavage process. In the immature egg the viscosity is high,

after maturation it drops. Upon fertilization it begins to rise again, to reach its maximum with the full development of the sperm aster. The viscosity drops again and continues low until the approach of cleavage. It thereupon rises again to drop only after completion of the first cleavage. Subsequent to the first cleavage the rhythmic appearance and disappearance of the asters within the blastomeres most probably indicate periodic successions of a process analogous to a jellying and liquefying of the cytoplasm.

The segmentation process may thus be explained as consisting essentially in a growth within the egg of two bodies of material through a gradual transformation of the cytoplasm. This transformation is associated with a change in the physical state of the protoplasm, two semisolid masses growing at the expense of the more fluid portions of the cytoplasm.

II. Cutting Experiments on the Segmenting Egg.

If it is true that the segmenting egg consists of two rather firm masses which are most fluid at their periphery, and if the physical state of the protoplasm is not affected in the process, one should be able to cut a segmenting egg into pieces without disturbing the cleavage plane. Cleavage should, therefore, proceed in such a manner as to complete the separation of what remains of the two bodies within each piece. This is what actually happens. Some experiments of Yatsu,[4] the results of which he made no attempt to explain, are in full accordance with mine and bear directly on this problem. Yatsu cut the eggs of *Cerebratulus* which were just beginning to segment (anaphase stage) into nucleated and non-nucleated fragments. He found that the cleavage furrow proceeded in its original plane irrespective of whether the fragments were nucleated or not. In Fig. 1, I have diagrammatically presented some of his results. Fig. 1 *a* represents a segmenting *Cerebratulus* egg being cut in a plane parallel to its long axis and to one side of the daughter nuclei. The original furrow persisted in the non-nucleated fragment (*b*) and

[4] Yatsu, N., Some experiments on cell-division in the egg of *Cerebratulus lacteus*, *Annot. zool. japon.*, 1908, vi, 267.

quickly completed its course in the nucleated fragment (*c*). Somewhat later the cleavage of the non-nucleated fragment (*d*) was also completed. Fig. 1 *e* represents a segmenting egg in which the cut

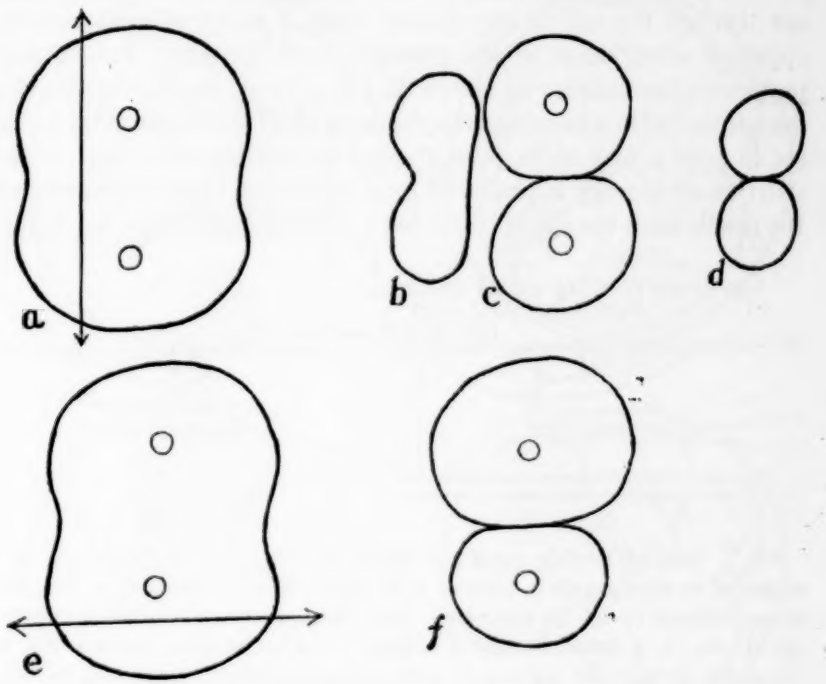

FIG. 1. A diagrammatic representation of Yatsu's results[4] on cutting the segmenting eggs of *Cerebratulus*. The direction of the cut is shown in *a*. The original cleavage furrow completed its course in the nucleated fragment *c* at the same time that it persisted in the non-nucleated fragment *b*. The furrow finally cut through the non-nucleated fragment in *d*. In *e* a cut was made across one end of the segmenting egg. The original furrow completed its course in *f* resulting in two unequal blastomeres.

was made at one end of the egg at right angles to its long axis. The original furrow persisted so as to divide the mutilated egg into two unequal blastomeres (*f*).

My cutting experiments were carried out mostly on the starfish egg, as sea urchins were very scarce during the summer of 1918.

The mature starfish egg averages 0.16 mm. (*i.e.* 160 μ) in diameter. The needles used for dissection averaged 10 μ in thickness at about 1 mm. from the tip and tapered gradually from there to a point far below 1 μ. With such a needle one can make a puncture or a clean cut through the egg in any desired spot or plane without causing apparent disturbance in the protoplasm of the egg. For cutting purposes glass needles as shown in Fig. 2 were used.[5] As the egg lies suspended in a hanging drop the end limb of the needle (Fig. 2 *a*) is set in such a way as to push the egg against the cover-slip. Constriction of the egg is produced by a continued upward pressure of the needle until the egg is cut in two. The operation does not neces-

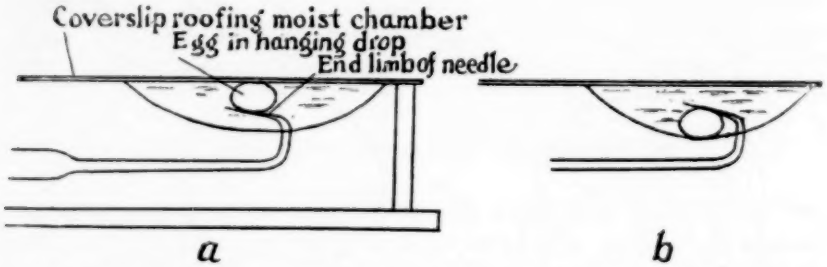

FIG. 2. Methods used for cutting an egg in two. *a*, side view of moist chamber magnified to show needle in position with its end limb so placed as to compress an egg between it and the cover-slip. Continued pressure of the needle cuts the egg in two. *b*, a second method of cutting an egg by bringing the end limb of the needle down on the egg so as to press the egg against the lower surface of the hanging drop.

sarily destroy the fertilization membrane which envelops the egg. The egg may also be cut in two on bringing it (Fig. 2 *b*) between the end limb of the needle and the lower surface of the hanging drop. Lowering the needle out of the drop in such a way as to give to the egg a rolling motion cuts the egg cleanly in two. This second method is not as satisfactory as the first for cases where one wishes to preserve the spatial relations of the egg contents, as the rolling motion produces churning movements within the cell.

Experiment 2.—(Figs. 3 to 7.) An *Asterias* ovum just beginning to segment and with the amphiaster in full development was cut

[5] Chambers, R., The microvivisection method, *Biol. Bull.*, 1918, xxxiv, 121.

in two in a plane diagonal to the cleavage furrow. The fresh surfaces caused by the cutting form films which prevent reunion of the pieces. The egg was in this way cut into two pieces each consisting of egg substance lying on both sides of the cleavage furrow.

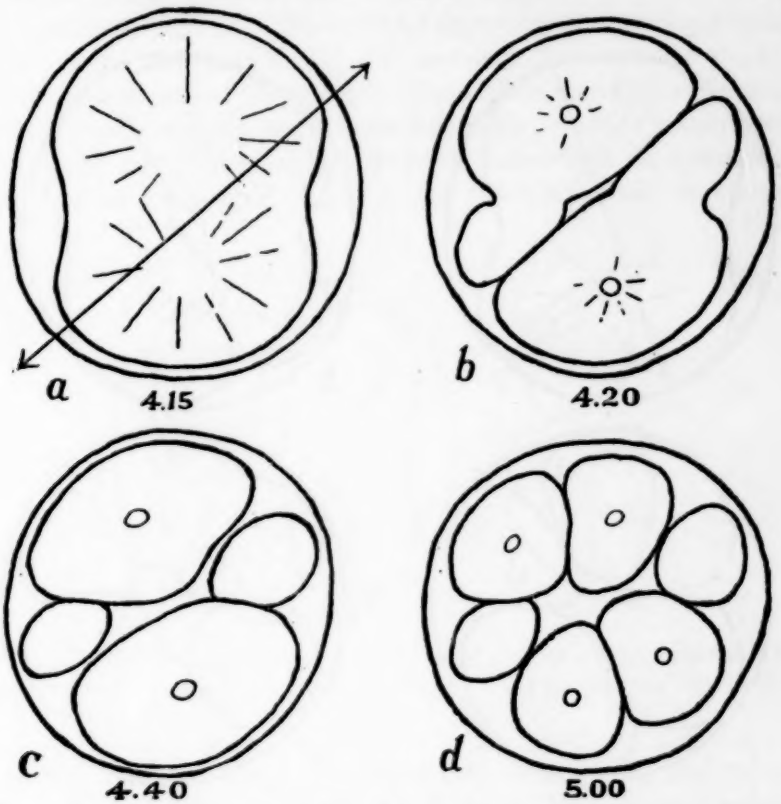

FIG. 3. Effect of a diagonal cut through an *Asterias* ovum beginning to segment in which the cut did not disturb the physical state of the ovum. *a*, operation performed at 4.15 p.m. *b*, 4.20 p.m., persistence of cleavage furrow in the original plane. *c*, 4.40 p.m., non-nucleated fragments pinched off. *d*, 5.00 p.m., nucleated fragments have segmented.

On one occasion the operation was performed on twelve eggs. In nine cases the original cleavage plane was maintained so that each piece pinched off a non-nucleated fragment normally belonging to the other blastomere. Two of them are illustrated in Figs. 3 *a* to *d* and 4 *a* to *d*.

In one case the cut was made at 4.15 p.m. (Fig. 3 *a*). 5 minutes later the cleavage furrow had progressed in the original plane (Fig. 3 *b*). At 4.40 it had completed its course so that each piece was divided into a small non-nucleated and a large nucleated fragment

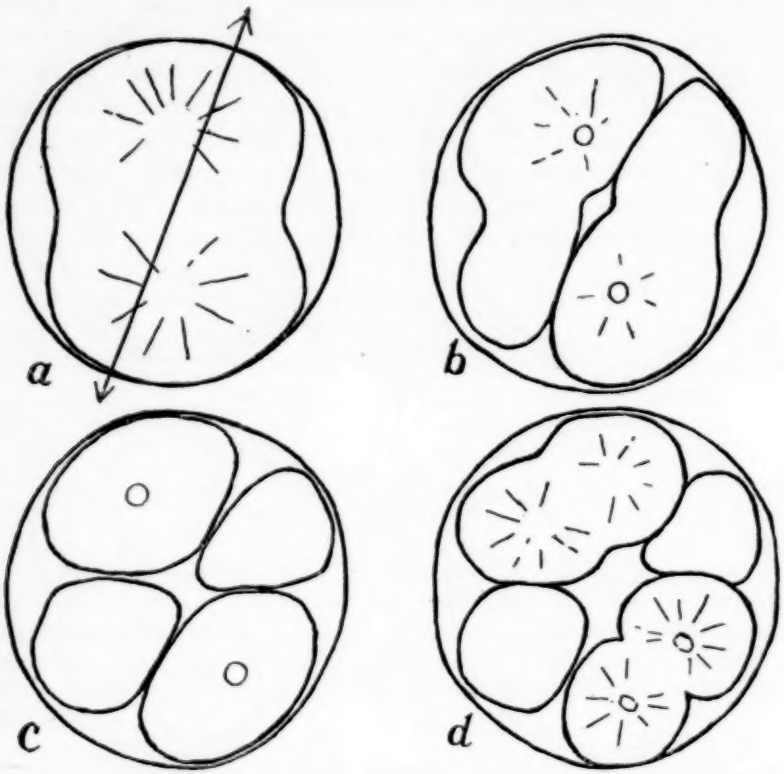

FIG. 4. Similar operation to that shown in Fig. 3 except that the diagonal cut is more nearly perpendicular to the cleavage plane with the result that larger non-nucleated fragments are pinched off by the cleavage furrow.

(Fig. 3 *c*). At 5 p.m. each of the two nucleated fragments or blastomere remnants had divided once (Fig. 3 *d*). 1 hour later they had divided once again. By the next morning the egg developed into a double blastula with the two non-nucleated fragments lying as inert masses within the fertilization membrane.

Fig. 4 *a* to *d* illustrates a similar case in which the non-nucleated masses are considerably larger than those depicted in Fig. 3. The similar behavior of one of the first two blastomeres in an egg is shown in Fig. 5 *a* and *b*.

In the remaining three cases the astral radiations faded out during the operation (Fig. 6 *a*). The original segmentation furrow gradually filled up and disappeared (Fig. 6 *b*) and each piece assumed the appearance of a normal blastomere. The nucleus then shifted so as to occupy a more central position in what one may term the reconstructed blastomere and further segmentation proceeded as if the ovum had not been operated upon (Fig. 6 *c* and *d*). This procedure always

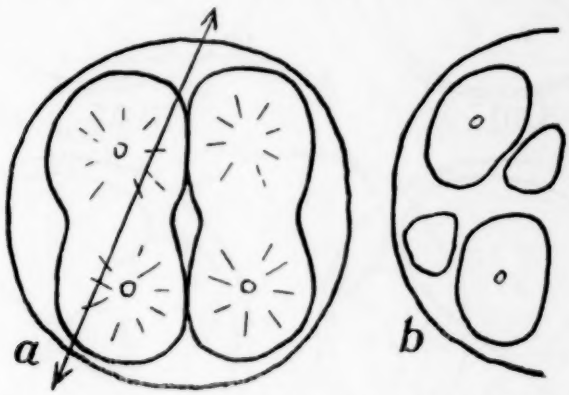

FIG. 5. Effect of a diagonal cut through one of the first two blastomeres of an *Asterias* ovum. *a*, egg showing direction of cut. *b*, cut blastomere a few minutes later.

occurred when the ovum was consciously rolled during the operation so as to produce a disturbance evidenced by a churning movement of the egg constituents.

A similar instance in the case of an *Arbacia* egg is shown in Fig. 7 *a* to *c*. A piece was cut from one pole of the amphiaster egg. In the process the piece was cytolyzed. The amphiaster in the remainder of the egg disappeared to reappear again in a new position with the result that two equal sized blastomeres were formed.

That mechanical disturbances may cause a reversal of a solid to a fluid state has already been shown.[3] This would make all the

protoplasm on each side of the cut merge into a single fluid mass. The nucleus then comes to occupy a central position. Normal mitosis takes place with the formation of an amphiaster and cleavage

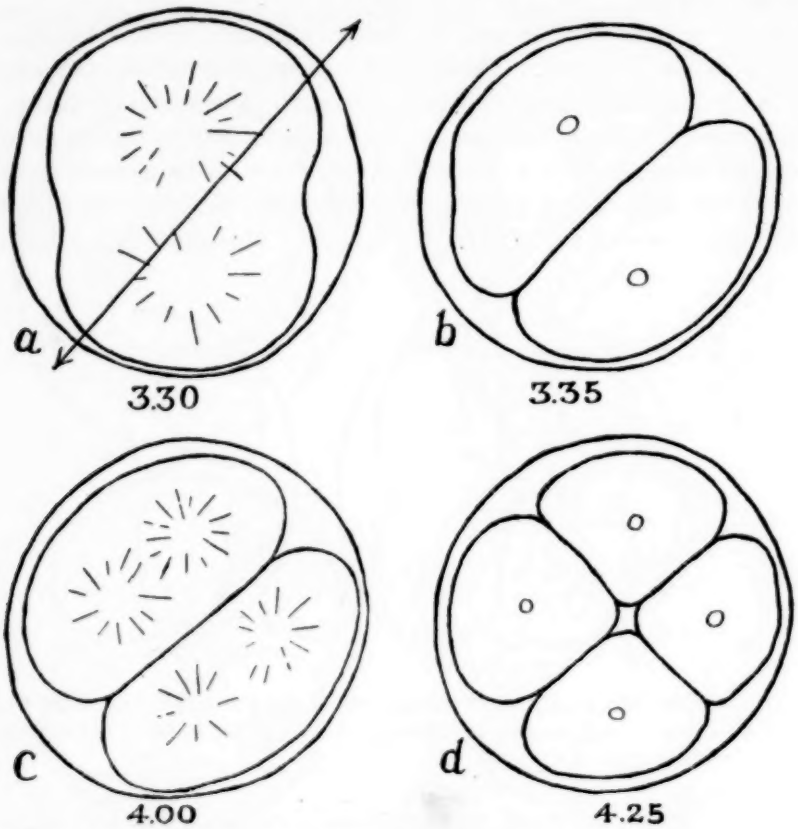

FIG. 6. Effect of a diagonal cut through an *Asterias* ovum in which the cut brought about a change in the physical state of the egg. *a*, operation performed at 3.30 p.m. *b*, 3.35 p.m., original cleavage furrow beginning to be obliterated. *c*, 4.00 p.m., an amphiaster formed in each of the two pieces produced by the cut. *d*, 4.25 p.m., four celled stage in which one cleavage plane was produced by the needle and the other by normal fission.

proceeds along the equator where the boundaries of the two asters are contiguous.

In the nine cases, in which the original cleavage plane persisted after the cutting process, the semisolid state about the two astral

centers was not disturbed. Each of the two pieces resulting from the cut, therefore, consisted of two unequal semisolid masses separated by a fluid area corresponding to the equator of the original egg. As this fluid area is incorporated into the two masses a furrow appears

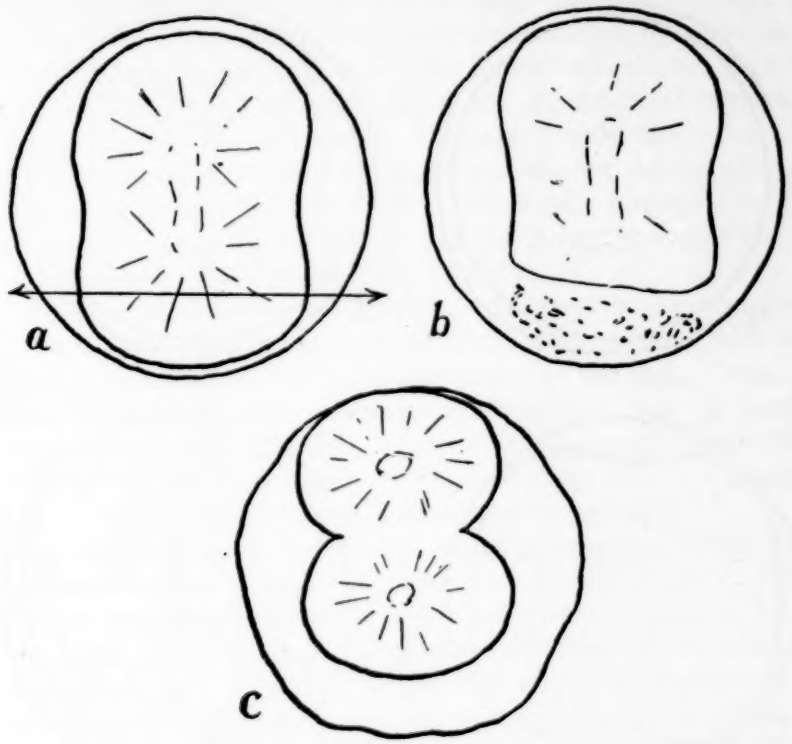

FIG. 7. Effect of cutting off a piece from one pole of an *Arbacia* ovum in the amphiaster stage. *a*, direction of cut. *b*, the piece cut off cytolized. The original shape of the remainder of the egg persisted for some time as the ovum of *Arbacia* is less pliable than that of *Asterias*. *c*, the reappearance of a new amphiaster resulting in the formation of two equal blastomeres.

which separates each piece into a larger nucleated and a smaller non-nucleated body.

The operated eggs were kept under observation until the gastrula stage, indicating that the operation had not destroyed the capacity of the egg for further development.

The following experiments are supplementary to the second. In all of them the results obtained are explicable on the basis of the existence, during cleavage, of reversible changes in the consistency of the cytoplasm.

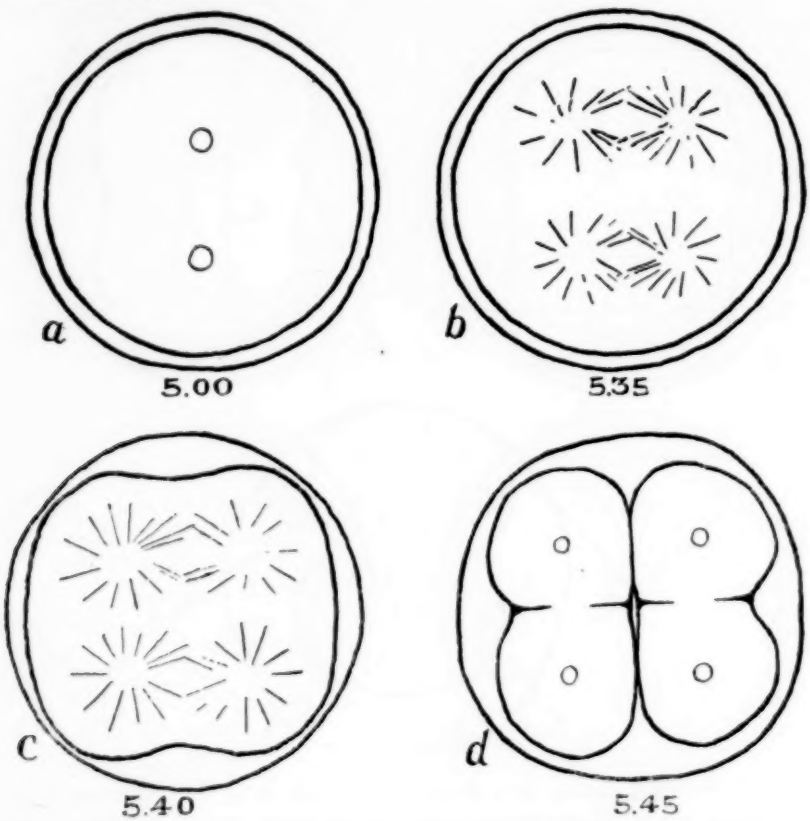

FIG. 8. Development of an *Asterias* ovum manipulated with a needle so as to suppress the first cleavage furrow. *a*, 5.00 p.m., disappearance of the amphiaster and obliteration of the cleavage furrow. *b*, 5.35 p.m., appearance of two amphiasters. *c*, 5.40 p.m., change in shape of the ovum with appearance of second cleavage furrow ahead of the first. *d*, 5.45 p.m., ovum cleaving into four blastomeres. (The ovum developed into a normal embryo.)

Experiment 3.—(Fig. 8.) In this case the first segmentation furrow was prevented from forming by tearing at the equator whenever it made its appearance. The progressive changes within the egg were

undisturbed. As soon as the amphiaster disappeared there was no longer a tendency for the furrow to form (Fig. 8 *a*). The two nuclei now lay in a fluid cytoplasm. Within half an hour after the suppression of the first segmentation furrow, an amphiaster developed about each nucleus preparatory to the next division. The two amphiasters lay side by side but remained distinct from one another, no connecting radiations being formed (Fig. 8 *b*). The formation of the two amphiasters resulted in the transformation of the egg substance into four semirigid bodies, the four asters. Cleavage furrows now extended into the fluid regions between the asters and divided the egg almost simultaneously into four blastomeres. The furrow corresponding to the second cleavage started to form and cut through the egg about a minute ahead of that of the first (Fig. 8 *c* and *d*).

This experiment may throw light on the nature of the segmentation in ova in which several nuclear divisions follow one another with no outward manifestation of the segmentation of the egg. After a certain period the ovum breaks up simultaneously into as many blastomeres as there are nuclei. This is the normal method in certain *Actinozoa* and can be artificially produced in many eggs by exposing them to various reagents, notably hypertonic solutions.[6,7]

The solidification associated with the aster formation divides the egg cytoplasm into a number of bodies each surrounding a nucleus. Between successive divisions the cytoplasm reverts to a more fluid state but its viscid nature may suffice in preventing the merging of neighboring areas. After a varying number of nuclear divisions with accompanying solidification periods furrows suddenly appear between these bodies and the ovum tends to break up at once into separate blastomeres. A differentiation of this type may possibly have taken

[6] Loeb, J., Investigations in physiological morphology. III. Experiments on cleavage, *J. Morph.*, 1892–93, vii, 253. Norman, W. W., Segmentation of the nucleus without segmentation of the protoplasm, *Arch. Entwcklngsmechn. Organ.*, 1896, iii, 106. Wilson, E. B., Experimental studies in cytology. I, *ibid.*, 1901, xii, 529. Lillie, R. S., Fusion of blastomeres and nuclear division without cell division in solutions of non-electrolytes, *Biol. Bull.*, 1902–03, iv, 164.

[7] Wilson, E. B., Experimental studies in cytology, II and III, *Arch. Entwcklngsmechn. Organ.*, 1902, xiii, 353.

place in the unsegmented *Chætopterus* embryos experimentally produced by Lillie.[8]

Experiment 4.—(Fig. 9.) Fig. 9 *a* to *e* depicts the case of an egg with the cleavage furrow just beginning in which the diagonal cut was incomplete so that the two pieces remained connected at one end of the cut. The original furrow persisted for a time during which it deepened considerably. 30 minutes after the cut had been made

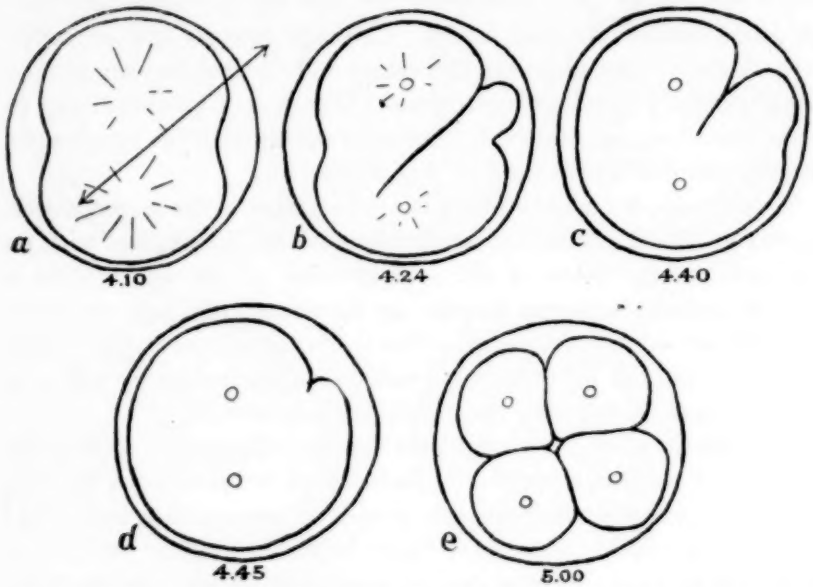

FIG. 9. Effect on an *Asterias* ovum of a deep cut which did not persist. *a*, operation performed at 4.10 p.m. *b*, *c*, and *d* show the egg respectively at 4.24, 4.40, and 4.45 p.m. Both the cut and the cleavage furrow disappear together with a reversal of the ovum from a semisolid to a more fluid state. *e*, 5.00 p.m., the ovum has divided into four normal blastomeres. (The ovum developed into a normal embryo.)

no sign of astral radiations were present and both the original segmentation furrow and the cut produced by the needle were being obliterated (Fig. 9 *c* and *d*). At 5 p.m. the egg had divided into four apparently normal blastomeres (Fig. 9 *e*) and was only slightly

[8] Lillie, F. R., Observations and experiments concerning the elementary phenomena of embryonic development in *Chætopterus*, *J. Exp. Zool.*, 1906, iii, 153.

behind the normal controls. By the next morning it had developed into a swimming blastula not to be distinguished from the normal controls.

The obliteration of the cut and of the furrow is consequent to a reversal of the egg cytoplasm from a semirigid to a more fluid state. The film projecting into the egg gradually merges into the liquid

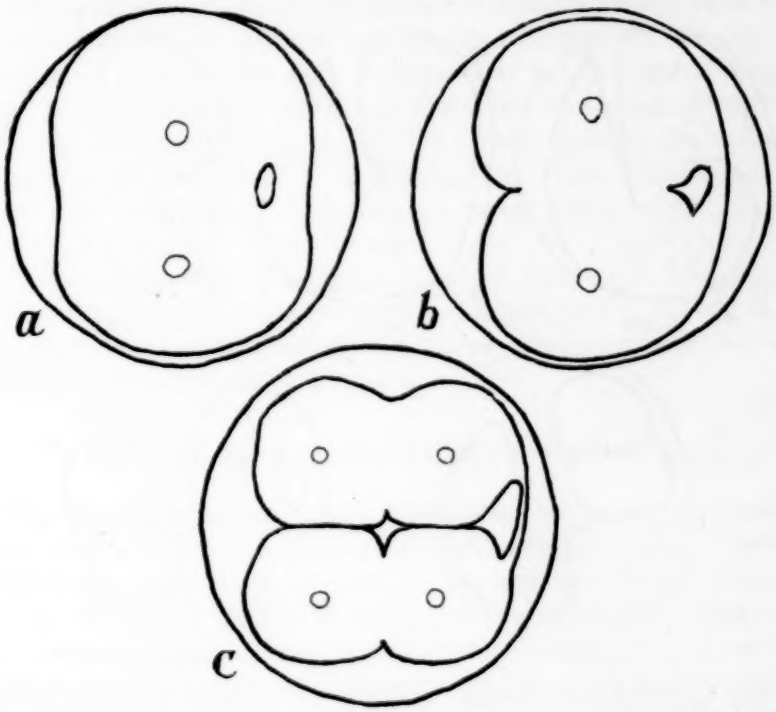

FIG. 10. Successive stages of an *Asterias* ovum showing persistence of a puncture made below the first cleavage furrow as it is beginning to form.

cytoplasm surrounding it and surface tension forces finally overcome the deformation of the egg. The egg now proceeded to divide into four blastomeres as in Experiment 3.

Experiment 5.—(Fig. 10.) This experiment demonstrates a peculiar property of the equatorial region during the formation of the cleavage furrow. A tear was made through the egg below the segmentation furrow (Fig. 10 *a*). The hole produced by the tear

remained open. The cleavage furrow continued its course beneath the hole leaving an outer margin as a bridge of protoplasm which connects the two blastomeres (Fig. 10 *b*, *c*). After several divisions of the egg the bridge thinned down in its middle until it broke through and the resulting strands were gradually drawn into the blastomeres from which they had projected.

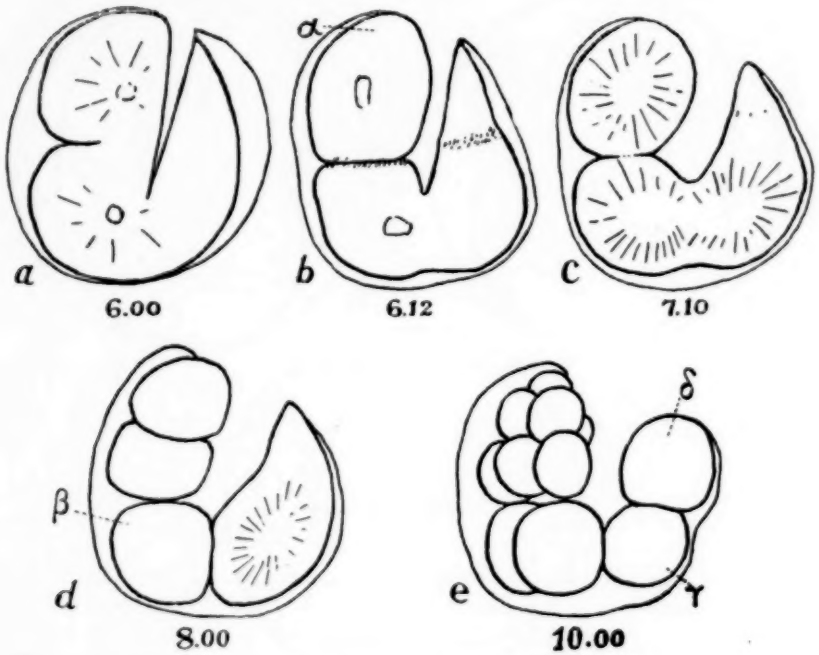

FIG. 11. Effect on an *Arbacia* ovum of a deep cut which persisted. For description of the results see text. Pigment granules collect in plane of original furrow.

Experiment 6.—(Fig. 11.) This experiment was performed on an *Arbacia* egg. An incomplete cut was made almost perpendicular to the cleavage furrow but to one side of the daughter nuclei. The furrow on the side away from the daughter nuclei became obliterated (Fig. 11 *a*). On the other side it continued its original course resulting in the pinching off of the nucleated Blastomere α (Fig. 11 *b*). The nucleus in the remainder of the egg shifted its position only slightly and

the amphiaster (Fig. 11 *c*), forming about it, resulted in a second unequal cleavage with the formation of Blastomere β (Fig. 11 *d*). The projecting piece of the egg above the obliterated furrow remained quiescent during these divisions and not until after the third unequal cleavage resulting in the formation of Blastomere γ in Fig. 11 *e*, did it become incorporated in Blastomere δ.

In this experiment the cut was probably made in the egg when the process for the first cleavage was too far advanced for the egg to retrace its course. The gash was therefore not obliterated and a very peculiar condition resulted in a succession of advances of the cleavage process about the gash. Blastomere α, being the earliest formed, segmented ahead of its fellows (Fig. 11 *d*). Blastomere β came next (Fig. 11 *e*). Unfortunately before Blastomeres γ and δ divided the egg died.

It is significant that Blastomere δ is larger than γ as evidently the former finally incorporated the hitherto inactive part of the egg that lay above that part of the original first cleavage furrow which lay on the right side of the gash (Fig. 11 *b*).

III. Concerning the Mechanism of Cell Division.

The changes in shape that an echinoderm egg undergoes during cleavage can be in part understood on the assumption that the astral formation is a solidifying process. It has long been known that at the time of cleavage the eggs of echinoderms, many worms, mammals, etc., become elongated,[9] the cleavage furrow forming in a plane at right angles to the long axis of the egg. As the furrow deepens, each resulting blastomere tends to assume the shape of a sphere (Fig. 12 *a*).

Nobody, however, has thus far been able to explain the cause of this elongation. The observations recorded in this paper may explain this phenomenon. The two spheres of solidification grow at the expense of all but possibly a small peripheral part of the fluid egg substance. The combined diameters of the two fully formed semisolid spheres are greater than the original diameter of the egg,

[9] Hertwig, O., Beiträge zur Kenntniss der Bildung, Befruchtung und Theilung des thierischen Eies, *Morph. Jahrb.*, 1876, i, 347. Gurwitsch, A., Morphologie und Biologie der Zelle, Jena, 1904.

and hence the egg must elongate. After elongation the surface of the egg seems to tear in the plane separating the two semisolid spheres. The periphery of the two asters of the amphiaster stage never becomes so firm as their interior. This may account for the observation of von Erlanger,[10] confirmed by Spek,[11] who described peripheral currents in the rapidly dividing nematode egg. In this egg peripheral currents flow from the two poles toward the equator and from there inward to the center of the egg. Spek suggests that such currents exist in all dividing eggs, and that they are easily visible in the nematode egg because of the great rapidity with which it segments. Conklin[12] described an inward flow of granules at the equator of the dividing *Crepid-*

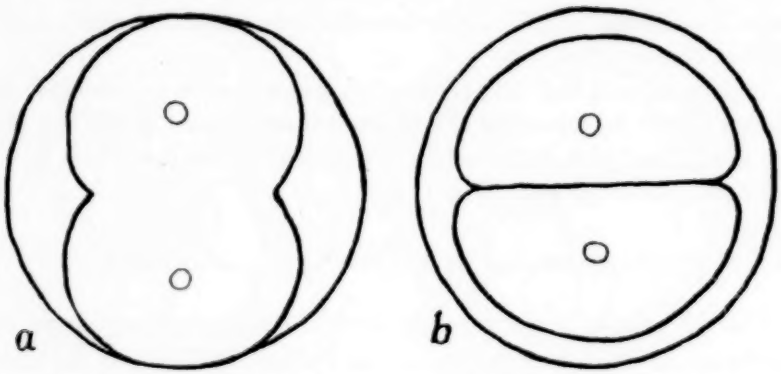

FIG. 12. Change in shape of an *Asterias* ovum (*a*) before and (*b*) after completion of the first cleavage furrow.

ula egg, and I[3] have observed a similar current, although a very slow one, in the sand-dollar egg.

Immediately after cleavage both of the two blastomeres are more or less spherical; but later, when they become more fluid, they are pressed against each other so as to be flattened at the plane of contact.

[10] von Erlanger, R., Beobachtungen über die Befruchtung und ersten Teilungen an den lebenden Eiern kleiner Nematoden, *Biol. Centr.*, 1897, xvii, 152, 339.

[11] Spek, J., Oberflächenspannungsdifferenzen als eine Ursache der Zellteilung, *Arch. Entwcklngsmechn. Organ.*, 1918, xliv, 5.

[12] Conklin, E. G., Protoplasmic movement as a factor of differentiation, *Marine Biol. Lab., Biol. Lect.*, 1899, 69.

Wilson,[7] in producing binucleate eggs by artificially obliterating the first cleavage furrow, noted that when this was caused by shaking, the resulting binucleate eggs retain the elongated shape (Fig. 13) characteristic of the egg in cleavage. During the ensuing pause (corresponding to the completion of the first cleavage and when

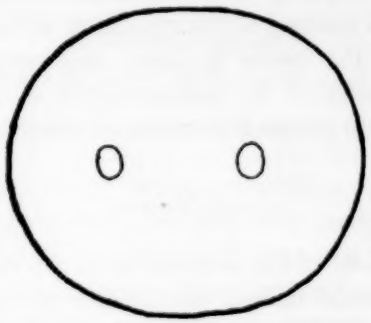

FIG. 13. Copy of Fig. 58 from Wilson[7] of *Toxopneustes* ovum immediately after shaking which caused obliteration of the first cleavage furrow.

FIG. 14. Copy of Fig. 34 from Wilson[7] of *Toxopneustes* ovum in which obliteration of the first cleavage furrow was produced by exposure to ether.

the astral radiations fade out preparatory to formation of a new amphiaster system) the egg becomes more nearly spherical. Evidently the shaking does not necessarily produce a reversal of the semisolid astral system to the more fluid state. As soon, however, as this occurs (in the ensuing pause) the egg resumes its spherical shape.

Wilson noted that the suppression of the cleavage furrow can also be produced by placing eggs, during their anaphase stage, in a 2.5 per cent ether solution. The astral radiations disappear and the resulting binucleate egg at once resumes the shape of a sphere (Fig. 14). This phenomenon may be comparable to the experiments illustrated in Figs. 6, 8, and 9 where the obliteration of the astral radiations follows a precocious reversal of the cytoplasm to the more fluid state. The suppression of the furrow in these cases seems to be primarily effected by the change in the physical state of the egg substance which, on reverting to a more fluid state, merges into a single spherical mass.

CONCLUSIONS.

1. The development of the amphiaster is associated with the formation of two semisolid masses within the more fluid egg substance.

2. The elongation of the egg during cleavage is possibly produced as a consequence of the mutual pressure of these two growing semisolid masses.

3. The division of the egg into two blastomeres consists essentially in a growth, within the egg, of two masses of material at the expense of the surrounding cytoplasm. When all the cytoplasm of the egg is incorporated in these two masses cleavage occurs.

4. After a certain period of time the semisolid masses revert to a more fluid state. In the eggs studied this normally occurs after the cleavage furrow has completed the separation of the two blastomeres. The formation of the furrow, however, may be prevented in various ways, upon which the egg reverts to a single spherical semifluid mass containing two nuclei.

5. An egg mutilated during its semisolid state (amphiaster stage) may or may not revert to a more fluid state. If the more solid state is maintained, the cleavage furrow persists and proceeds till cleavage is completed. If the mutilation causes the egg to revert to the more fluid state the furrow becomes obliterated and a new cleavage plane is subsequently adopted.

6. The nuclei of eggs in the semifluid state are able to alter their positions. In semifluid mutilated eggs the nuclei tend to move to positions which may assure symmetry in aster formation and cleavage.

A CHANGE IN THE BAR GENE OF DROSOPHILA INVOLVING FURTHER DECREASE IN FACET NUMBER AND INCREASE IN DOMINANCE.*

By CHARLES ZELENY.

(From the Zoological Laboratory of the University of Illinois, Urbana.)

(Received for publication, July 15, 1919.)

As part of a study of the factors controlling eye facet number in *Drosophila melanogaster*, selection has been carried on with a view to the determination of the germinal changes appearing during the course of selection. One of the most interesting of these changes occurred during the second generation of downward selection in the white bar stock. An exceptionally "low" male of this generation was shown to possess a changed gene with a marked increase in dominance. This individual appeared on November 20, 1917, and had but 19 facets, while the mean of its 62 brothers was 82.6 facets with a range of 41 to 134. With class sizes such that the range of a class is 10 per cent of its mean this represents a departure of −16.80 10 per cent class units from the mean of the males of the unselected bar stock, while the departure of the brothers of the mutant is only −3.44 units. The new stock derived from the third hybrid generation of a mating of the mutant with a sister possesses the characteristics of the original male, and at the present writing (May 10, 1919) has remained without change for nearly 18 months except for the appearance of a few additional mutants.

The name ultra-bar has been given to the new character. Its symbol, B^u, indicates that ultra-bar is a dominant allelomorph of bar. Full-eyed (wild-type) females at 27°C. have an average facet number of 810.6, bar females of the second low selected generation have 61.8 facets, and ultra-bar females 22.0 facets. The corresponding numbers for males are respectively 849.8, 75.6, and 23.0.

*Contribution from the Zoological Laboratory of the University of Illinois, No. 140.

The change in dominance as a result of the mutation is very striking. A cross between a 35.2 facet bar stock of the twenty-fourth generation of the low selection line and an 810.6 facet full-eyed stock gives 399.9 facets in the females, while a cross between the 22.0 facet ultra-bar and the 810.6 facet full gives only 36.5 facets. Ultra-bar thus has a much greater degree of dominance than bar. This is further shown by the cross between the 22.0 facet ultra-bar stock and the 61.8 facet bar stock of the second generation of the low selection line which gives 26.3 facets. Calculated on the basis of 10 per cent class units the ultra-bar has pulled the full-eye down 85 per cent of the distance between the two, while bar has pulled it only 23 per cent. Likewise ultra-bar pulls bar down 85 per cent of the distance between the two, while bar pulls ultra-bar up only 15 per cent.

That the factor for the new character is located in the X chromosome is shown by the reciprocal matings between ultra-bar and bar. Ultra-bar females mated with bar males give ultra-bar males, while the reciprocal matings give bar males.

In order to determine the locus of the new factor the crossing over test was applied. If the new factor is at a different locus from that of bar, crossing over should take place between the two. A mating between ultra-bar and full-eye should then give some bar males in the second hybrid generation, the percentage of such individuals depending upon the distance between the locus of bar and that of ultra-bar. In case, however, the new locus is identical with that of bar there should be no bar males in this generation.

Crosses between ultra-bar females and wild full males give in the second hybrid generation only three males which are not ultra-bar as opposed to 1,238 ultra-bar males. These three males have 88, 51, and 60 facets respectively, all being within the range of bar-eye. On the basis of crossing over this would mean that the locus of the new factor is only 0.24 units from the bar locus on the chromosome map. Unfortunately one of the three males was overetherized. Breeding tests of the other two show a degree of dominance over full which is different from that of bar and they therefore cannot be the result of crossing over. They apparently represent a new mutation.

The most reasonable conclusion to be drawn from these tests is that the locus of ultra-bar is the same as that of bar or that it is

so close to bar as to act as a unit with it for all demonstrable cases. The present case is then an instance of a second mutation in the same germinal material and in the same direction as a previous mutation.

The ultra-bar mutant adds to the series of germinal changes which have been made out in the case of eye-facet number. As previously reported germinal changes have been obtained which involve accessory factors in other than the sex chromosome, some of these producing an effect of large degree and others of small degree. Reverse mutations of the bar stock to full have also been observed. The case described in the present paper is a further step in the same direction as bar. In addition to the further decrease in facet number ultra-bar has a much greater degree of dominance than bar. Furthermore it occurred in the direction of selection, appearing in the low and not in the high selection line.

ADRENALIN IN ANNELIDS.

A Contribution to the Comparative Study of the Origin of the Sympathetic and the Adrenalin-Secreting Systems and of the Vascular Muscles Which They Regulate.

By J. F. GASKELL.

(From the Laboratory of Pathology, St. Bartholomew's Hospital, and the Physiological Laboratory, Cambridge, England.)

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The intimate physiological relationship between the sympathetic nervous system and the adrenalin-secreting cells of the medulla of the suprarenal body is now thoroughly established. All the actions of the sympathetic system can be imitated by adrenalin, and failure of the adrenalin supply, such as takes place in Addison's disease, causes failure of the proper action of the sympathetic nerves. The morphological relationship of the two types of cell, the sympathetic nerve cell and the adrenalin-secreting cell, are also equally intimate. The adrenalin-secreting cell is always identifiable in mammals by its chrome-staining reaction. Elliott[1] has brought forward strong evidence that the innervation of the medullary cells of the suprarenal body is a direct one by the medullated connector or tract fibers of the splanchnic nerves. Such an innervation corresponds to the white rami communicantes which connect with the sympathetic nerve cells in their various ganglia.

In the higher mammals the sympathetic ganglia do not usually contain any adrenalin-secreting cells, the latter being concentrated almost entirely in the special tissue of the suprarenal medulla; but in the lower forms of vertebrates the two tissues are both widely distributed and intimately connected. In amphibia, for instance, islets of chrome-staining tissues are to be found in every sympathetic ganglion,

[1] Elliott, T. R., *J. Physiol*, 1913, xlvi, 285.

and Smirnow[2] has shown that such cells are innervated by nerve fibers which are otherwise indistinguishable from those which run to the sympathetic nerve cells lying with them. Further back in the vertebrate kingdom the distribution becomes still more diffuse and the sympathetic nervous system becomes less and less defined and more and more replaced by a diffuse system of chrome-staining cells arranged segmentally throughout the body.

A definite sympathetic nervous system has been long known in the case of the elasmobranch fishes, but it is only comparatively recently that the researches of Giacomini and others have definitely demonstrated its presence in the other groups of fishes. Giacomini finds a definite double sympathetic chain present in the Dipnoi,[3] but in the Ganoidei[4] and Teleostei[5] he was able to find only an irregular system of nerve cells distributed along the cardinal veins. In the cyclostomes[6] the representatives of the sympathetic nervous system are still more scanty, being certain nerve cells which are occasionally to be found around the cardinal veins. In all these groups of fishes there is again an intimate relationship between the sympathetic nerve cells and diffusely distributed chrome-staining cells. The presence of adrenalin in the latter cells was demonstrated by Vincent in the elasmobranchs,[7] and in a previous paper I[8] gave reasons for believing that the chrome-staining tissue of the cyclostome, *Petromyzon fluviatilis*, also contained adrenalin. An extract of such tissue caused a rise of blood pressure in the cat, which was in every way similar to that caused by a small dose of adrenalin. The conclusion may therefore be drawn that chrome-staining tissue, wherever found among vertebrates, secretes adrenalin, and the presence of a sympathetic nervous system and an adrenalin-secreting system may be considered to have been established throughout the vertebrate kingdom. The two systems are in every animal most intimately connected physio

[2] Smirnow, A., *Arch. mikr. Anat.*, 1890, xxxv, 416.
[3] Giacomini, E., *Atti Accad. Lincei, Rendiconti*, 1906, xv, series 5, 394.
[4] Giacomini, E., *Monitore zool. ital.*, 1904, xv, 20.
[5] Giacomini, E., *Monitore zool. ital.*, 1902, xiii, 183.
[6] Giacomini, E., *Monitore zool. ital.*, 1902, xiii, 143.
[7] Vincent, S., *Proc. Roy. Soc. London*, 1897, lxi, 64.
[8] Gaskell, J. F., *J. Physiol.*, 1912–13, xliv, 59.

logically and anatomically. The work of Kohn[9] has also shown how closely their evolution in the vertebrate is paralleled by their embryological development in the mammal.

A search for the origin of the two systems must therefore be pursued in the invertebrate kingdom. If it is allowed that the presence of a yellow coloration after fixation with a chrome salt in certain cells, which have been called chromaffin cells, occurs among vertebrates only in cells which contain adrenalin, the presence of such a reaction in cells of invertebrates is presumptive evidence that the latter are also adrenalin-secreting cells. The first discovery of chromaffin cells in the invertebrate was made by Poll and Sommer[10] who described the occurrence of the reaction in certain cells of the central nervous system of the leech, *Hirudo medicinalis*. Poll[11] has since extended this observation by finding similar cells in certain other annelids. The only other observation is that of Roaf and Nierenstein[12] who extracted an adrenalin-like body from certain tissues lying in the walls of the branchial chamber of the mollusk, *Purpura lapillus*. Subsequently Roaf[13] located this secretion to certain cells which gave a chromaffin reaction. As the Mollusca are not held to be on the direct line of vertebrate descent attention has been confined to the annelid kingdom.

A representative selection of annelids was examined at the Zoological Station of Naples, and a detailed description of the results has been given in my paper published in 1914.[14] Among the Hirudineæ chromaffin cells have been found in the ganglia of the central nervous system in all species investigated. Seventeen different animals of the polychæte group were examined but in fifteen of these no trace of chromaffin cells could be found; in the remaining two, *Aphrodite aculeata* and *Eunice gigantea*, small chromaffin cells were present. *Lumbricus herculeus*, the only oligochæte investigated, also possessed

[9] Kohn, A., *Arch. mikr. Anat.*, 1903, lxii, 263.

[10] Poll, H., and Sommer, A., *Arch. physiol.*, 1903, 549.

[11] Poll, H., in, Hertwig, O., Handbuch die vergleichende und experimentelle Entwicklungslehre der Wirbeltiere, Jena, 1906, iii, pt. 1, 603.

[12] Roaf, H. E., and Nierenstein, M., *J. Physiol.*, 1907, xxxvi, p. v.

[13] Roaf, H. E., *Quart. J. Exp. Physiol.*, 1911, iv, 89.

[14] Gaskell, J. F., *Phil. Trans. Roy. Soc. London, Series B*, 1914, ccv, 153.

them. Wherever they were found these chromaffin cells were always constant in number and similar in position in the ganglion, six being always present arranged in three groups of two each, a ventral and two lateral groups. The two cells of the ventral group were usually the largest. The size of the cells varied greatly in the different animals, being small in the two polychætes and reaching the largest size in *Hirudo medicinalis*, which was therefore selected for further investigation.

In this animal the two ventral cells are of very large size; they have been called colossal or giant cells by Retzius[15] and others. The lateral group on each side consists of two smaller cells which lie respectively just posterior to the anterior and posterior lateral nerves; their positions are indicated in Fig. 1. These six cells have all the appearance of nerve cells, possessing processes which run out in the lateral nerves and staining similarly to the other nerve cells of the ganglion. It was found that the six cells could be very clearly demonstrated by staining the freshly excised ganglion with methylene blue, and then irrigating the preparation mounted in water under a cover-slip with a dilute bichromate solution; all the nerve cells became bleached by this process with the exception of the six chromaffin cells which retained the blue stain. The probable explanation of this reaction is that the bleaching action of the chrome salt is prevented in the chromaffin cells by its combination with the chrome-staining substance, thus protecting the methylene blue stain. The nerve cells of the ganglion of the leech are divided into groups, shut off from one another by septa, whose arrangement is shown in Fig. 1. The individual nerve cells are unipolar and hang free in their particular compartment lying in a clear nutritive fluid; their relative positions are therefore liable to alterations in the compartment, for instance the two giant cells can be moved about freely by pressure on the cover-slip in a suitable preparation. The nerve cord itself is suspended in a blood space known as the ventral sinus, the thin sheath of the ganglion intervening only between the compartments in which the nerve cells lie and the surrounding blood. It is quite possible that an interchange takes place between the fluid in which the nerve cells are suspended and the blood

[15] Retzius, G., *Biol. Untersuch.*, 1891, ii, 13.

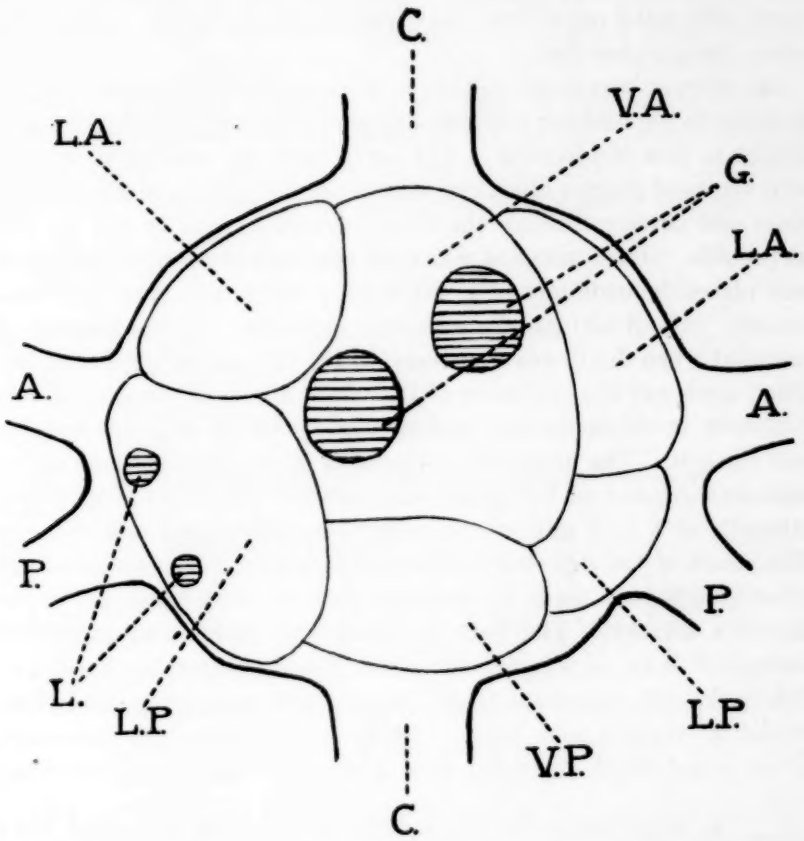

FIG. 1. Diagram of a ganglion of the leech showing the compartments in which the nerve cells are grouped and the position of the chromaffin cells in them. The limits of the various cell groups are shown, with regard to the ventral side of the ganglion on the right and center, with regard to the dorsal side on the left. *G.*, the two giant cells lying in the ventral anterior group. *L.*, the lateral cells lying in the dorsal portion of the posterior lateral groups. *V.A.*, the ventral anterior group. *V.P.*, the ventral posterior group. *L.A.*, the lateral anterior group. *L.P.*, the lateral posterior group. *C.*, the connectives. *A.*, the anterior nerves. *P.*, the posterior nerves.

of the sinus, and that any secretion, such as that of adrenalin in the chromaffin cells, could thus reach the circulation of the segment in which the ganglion lies.

An attempt was made to obtain an extract of the central ganglia in order to see whether any physiological reaction could be obtained similar to that of adrenalin. The nerve cords of a number of leeches were removed under a dissecting microscope by opening up the ventral sinus and cutting through the lateral nerves as near to the ganglia as possible. Each ganglion was then cut away from its connectives and placed immediately in a watch-glass which was kept in a desiccator. About 400 ganglia were thus collected. As the amount of material when dried was extremely small, the physiological test decided upon was the inhibition of the virgin uterus in the cat. I was fortunate in obtaining the assistance of Dr. H. H. Dale in carrying out the test. The uterus was suspended in Ringer's solution and a sufficient amount of histamine was added to the bath to give it a strength of 1 in 3 millions, in order to produce tone and rhythm. The result of the experiment is shown in Fig. 2. An extract of the dried ganglia was made by grinding them up with sand in 2 cc. of Ringer's solution. The bath in which the uterus was suspended contained 50 cc. of Ringer. At *A* the 2 cc. of extract were added to this bath, with the result that a distinct lowering of tone and diminution of rhythm took place. Similar experiments with extremely dilute solutions of adrenalin gave a much stronger inhibition when $\frac{1}{2,000}$ mg. was added to the 50 cc. bath, but a smaller inhibition when $\frac{1}{20,000}$ mg. was added; the extract therefore contained an amount of adrenalin lying between $\frac{1}{5,000}$ and $\frac{1}{10,000}$ mg. This experiment supports the conclusion that the chromaffin reaction of the six nerve cells in the ganglion of the leech is due to the presence of adrenalin in them. It confirms a statement of Biedl that he has been able to obtain the biological tests for adrenalin from these cells. I have, however, been unable to find any detailed description of his experiments.

The structure of these adrenalin-containing cells justifies the conclusion that they are nerve cells, and the question arises whether they innervate some special musculature which is susceptible to the action of adrenalin. That is to say, are they the representatives of the sympathetic cells of the vertebrate as well as the representatives of the adrenalin-secreting cells of the suprarenal medulla? If this is so, in this primitive stage of development, such a nerve cell not only

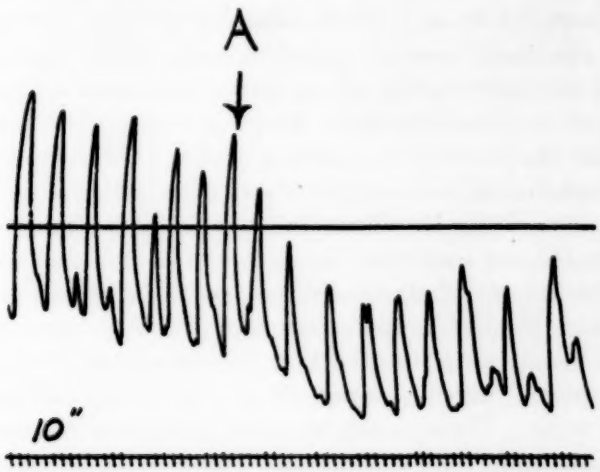

FIG. 2. The action of an extract of leech ganglia upon the virgin uterus of the cat. The isolated uterus was suspended in a bath of 50 cc. of Ringer's solution, to which histamine had been added to produce a strength of 1 in 3 millions; a good tone and rhythm were thus obtained. At *A* the extract of 400 leech ganglia ground up with sand in 2 cc. of Ringer's solution was added to the bath. A diminution in both tone and rhythm was produced. Time markings at intervals of 10 seconds.

regulates its peripheral musculature by direct nervous action but also provides the internal secretion which is necessary for the proper action of its nervous impulses. Later developments have caused a differentiation from this primitive state, so that two separate types of cell have arisen, one of which has become the adrenalin-secreting cell, the other the sympathetic nerve cell. Kohn[9] considers that in the early mammalian embryo the two types of cell arise from a common mother cell, and may develop into either chromaffin cells or

sympathetic nerve cells. He has therefore called the chromaffin system the paraganglion system, in order to point out its intimate relationship to the ganglia of the sympathetic chains. There is thus a close parallel between the condition in *Hirudo* and the early embryological development of these tissues in the mammal. The connections of the two types of cell with the central nervous system in the vertebrate, which have already been referred to, also support the theory that they have been derived from a common ancestral cell, for both are supplied by medullated connector or tract fibers which run out from the central nervous system to connect with them.

During the investigation of the nervous systems of the various members of the annelid groups, a vascular system with definite muscular walls was found always to exist when chromaffin nerve cells were present, but to be absent when such nerve cells were also absent. The members of the Hirudineæ investigated all possessed muscular walled vessels, and *Lumbricus* has, as is well known, similar muscular "hearts" which are rhythmically contractile. *Eunice gigantia*, one of the two members of the polychæte group in which chromaffin nerve cells were found, possesses a short portion of vessel at the base of each of the branchiæ, which is suspended on a mesentery and has definite muscular walls. These vessels by their contraction drive blood into the branchiæ to be oxygenated. Contractile muscular walled vessels are therefore present in this animal. The vascular system of *Aphrodite* could not be thoroughly investigated owing to lack of material; the question of the presence of vessels with muscular walls could not therefore be decided. In all the other members of the polychæte group, which possessed any definite vessels at all, no sign of any muscular tissue could be found on their walls. It appears that, wherever vascular muscle is present in the Annelida, chromaffin nerve cells are also to be found in the central nervous system, and that these cells are adrenalin-secreting; on the other hand, if no vascular muscle is present no chromaffin cells exist. These facts support the view that the chromaffin nerve cells innervate the vascular muscles.

Further investigations were made to attempt to discover the nature of the innervation of the vascular muscles in the leech, and also their reaction to adrenalin. The nervous system and vascular system were investigated in detail and the results are described in full in my pre-

vious paper.[14] It was found that a small branch of the anterior nerve could be traced directly to the wall of the lateral vessel in each segment, and that this nerve was formed of processes of cells situated in the central segmental ganglion. A branch of the posterior nerve also appeared to run to the same vessel, though its complete continuity was not established. The vascular muscles are therefore innervated by processes of cells situated in the central ganglion, the arrangement being a segmental one.

The lateral vessels were found to have a contractile rhythm with an average beat of about six to eight contractions per minute, and this rhythm was independent of nervous control, for it continued for many hours after complete section of all the lateral nerves. Rhythmical contraction is, in the vessels of the leech, the property of the vascular muscle itself; that is to say, the beat is myogenic not neurogenic.

The effect of section and stimulation of the lateral nerves upon the contractile rhythm of the lateral vessels was also investigated. The study of the vascular beat was much facilitated by the use of curare, which completely paralyzes the longitudinal and circular muscles but does not affect the vascular muscles. The dose required is large, being about 0.2 cc. of a 1 per cent solution, but the differentiation of the two types of muscle by the action of curare is a complete one. Attempts were made to obtain some mechanical method of recording the vascular beat but nothing sufficiently delicate could be devised. The beats were therefore observed under a dissecting microscope and recorded by a key signal on a revolving drum. As the animal had to be kept in Ringer's solution throughout the experiments, stimulation was brought about by means of Lucas'[16] electrode applied to the main nerve chain, the only nerve left intact being the one it was desired to stimulate. It was found that section of the anterior nerve with the posterior nerve intact always caused definite slowing of the rate of beat, while stimulation of this nerve with the posterior nerve divided caused acceleration. The anterior nerve thus contains accelerator fibers to the vascular muscle. Section of the posterior nerve with the anterior nerve intact caused quickening of the rate, while stimulation of this nerve with the anterior nerve divided in the cura-

[16] Lucas, K., *J. Physiol.*, 1913, xlvi, p. xxxii.

rized leech had no effect, but in the decapitated animal caused marked slowing of the rate. The posterior nerve therefore contains fibers which are inhibitor to the vascular muscle. The acceleration effect produced by stimulation of the anterior nerve is abolished by the injection of ergotoxin. The vascular system is thus definitely under the control of the central nervous system, the arrangement being a segmental one, and the length of the vessel lying in any segment being controlled by cells in the ganglion of that segment. The accelerator fibers run in the anterior nerve, and their action is abolished by ergotoxin in the same way as is the case with the mammalian sympathetic system. These accelerator fibers have been definitely traced to the muscle and are very probably the processes of the chromaffin nerve cells, which therefore control the vascular muscle in the leech in a similar way to its control in the vertebrate by the sympathetic system. The inhibitor fibers run in the posterior nerve and are also processes of cells in the central ganglion. Their action is abolished by curare. They control the vascular muscle in a manner strictly comparable to the control of the vertebrate heart by the vagus nerve.

The nerve supply of the vascular muscles is therefore a double one which is strictly comparable to the double supply of the vertebrate heart by the sympathetic and vagus systems. The power of rhythmical contraction is an intrinsic property of the vascular muscle itself, to which the two nerves act as regulators. In the primitive form found in annelids, the heart beat is therefore myogenic in origin, but is regulated by the control of the nervous system.

The action of adrenalin was tried both on the longitudinal and circular voluntary muscles and on the vascular muscles. Experiments on the voluntary muscles of the leech were difficult, and were supplemented by similar experiments on *Lumbricus*. No effect could be obtained in these muscles in either animal by the application of adrenalin if it was applied in neutral solution; with the usual acid solution in the form of hydrochloride the acidity of the solution was always sufficient to cause a contraction. Adrenalin borate was found to be a form suitable for the purpose. It was applied directly to a lateral vessel of the leech, after exposing it under Ringer's solution, by injection into the loose tissue lying around the vessel. The effect of the injection of one drop of a solution in Ringer of adrenalin borate

of a strength of 1 in 10,000 is shown in Fig. 3. A beat of an average interval of 14.5 seconds was accelerated to a beat of 9.4 seconds. Later this segment stopped in systole, and the neighboring segments increased their rate of beat. Adrenalin therefore causes a marked acceleration and, if in sufficient strength, it further causes complete contraction which abolishes rhythm altogether. The vascular muscle is sensitive to the action of adrenalin while the voluntary muscle is not; and the action on the vascular muscle is strictly comparable to that on the vertebrate heart.

In order to carry still further the close physiological relationship which had become apparent between the muscles of the vessels of the leech and that of the vertebrate heart, the actions of atropine and muscarine were also tried. Atropine was found to cause an accele-

FIG. 3. The action of adrenalin borate on a lateral vessel of the curarized leech. At the signal mark one drop of a solution of adrenalin borate of a strength of 1 in 10,000 was injected into the tissues around the vessel. A beat with an average interval of 14.5 seconds was accelerated to a beat with an average interval of 9.4 seconds. Time marking in seconds.

ration which gave the fastest rhythm ever observed, the average interval between beats dropping to 4.2 seconds, a rate of over fourteen beats per minute. The efficiency of the beat also became maximal. Muscarine in strong solution caused complete cessation of beat in diastole; in dilute solution it caused weakening and slowing of the beat. The complete cessation of the beat caused by muscarine can be removed by a subsequent injection of atropine, if the atropine is injected soon after the beat has ceased. The vessel gradually resumes a rhythmical contraction. If, however, the muscarine has been allowed to act for some time, atropine has no longer the power to recover the beat. These actions of atropine and muscarine are strictly comparable to their actions on the vertebrate heart.

The leech *Hirudo medicinalis* thus possesses a very definite type of vascular muscle clothing certain of its vessels which is rhythmically

contractile and has physiological actions in every way comparable to those of the vertebrate heart. It is controlled by similar nerves and reacts to the drugs adrenalin, atropine, muscarine, and curare in an identical way. The two main vessels, which lie in the extreme lateral position in the body just under the longitudinal muscle layers, are also similar in function. Their chief purpose is to drive blood into an extensive capillary network which lies in the skin and is respiratory in function. The condition in *Eunice gigantea* is probably a primitive form of that in the leech. The segmentally arranged musculature which surrounds a short portion of the vessel lying at the base of each branchia has become more diffusely spread and has fused to form one continuous contractile vessel on each side. In *Eunice* again the function of these "hearts" is entirely branchial. The growing around of the lateral folds of the invertebrate to form the ventral surface of the vertebrate, which is hypothecated in the theory of the origin of vertebrates brought forward by W. H. Gaskell,[17] would carry with them the two lateral vessels. They would thus become midventral and would lie in the position of the two vessels from which embryologically the vertebrate heart is formed. The physiological function of such a heart would be always branchial from its earliest origin in the annelid kingdom.

The formation of a specialized vascular muscle immediately demanded the formation of special nervous and chemical regulators to this muscle; we therefore find a segmental nervous control already established in annelids for the vascular muscle of each segment, and a specialized cell which secretes the necessary internal secretion; namely, adrenalin. In this primitive condition the secretion of adrenalin is a function of nerve cells, constant in number, which are situated in the segmental ganglion and are also in all probability the nervous regulators of the vascular muscle. These cells therefore represent the common ancestors of the sympathetic and of the adrenalin-secreting systems of the mammal. In the course of evolution the two functions have become separated, and two distinct types of cell have arisen, one of which is purely secretory and the other purely nervous. In the earliest vertebrates the secretory system is chiefly

[17] Gaskell, W. H., The origin of vertebrates, London, 1908.

in evidence, but it is here most intimately connected with the ganglia and trunks of the posterior nerves, an arrangement strictly comparable with Ônodi's[18] description of the early development of the mammalian sympathetic system. The evolution in the vertebrate kingdom of the two now separate systems takes the form of a steadily increasing development of the nervous or sympathetic type of cell and a relative diminution and concentration of the secretory type. The final condition reached in the mammal is a widely distributed complex sympathetic nervous system, with a complete concentration of the secretory system into the medullary tissue of the suprarenal capsules.

The emigration from the central nervous system of the cells which secrete adrenalin took place at the same time as that of the nerve cells of the sympathetic system; their close association and similarity of nervous control is still clearly seen in animals as high in the vertebrate scale as the amphibia, where chromaffin cells are incorporated in every sympathetic ganglion.

CONCLUSIONS.

1. The sympathetic nervous system and the adjuvant adrenalin-secreting system are found in their earliest form in the annelid kingdom, and consist of cells situated in the central nervous system which are the common ancestors of both, and which are both secretory and nervous in function.

2. These cells are developed in the annelid kingdom parallel with the development of a contractile vascular system, which possesses muscles comparable in physiological actions with the muscle of the vertebrate heart.

3. This vascular muscle is regulated by the processes of the common ancestral cells as well as by their secretory activity.

4. In the primitive form contractile rhythm is an intrinsic property of cardiac muscle; its nerve supply regulates the rhythm, it does not initiate it. The beat is therefore myogenic, not neurogenic.

5. The contractile vascular system of annelids is mainly branchial in function. The vertebrate heart has been derived from it by the growing around of the lateral body folds to form a new ventral surface.

[18] Ônodi, A. D., *Arch. mikr. Anat.*, 1885–86, xxvi, 553.

ELECTRIFICATION OF WATER AND OSMOTIC PRESSURE.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I. INTRODUCTION.

When a watery solution is separated from pure water by a strictly semipermeable membrane we call osmotic pressure the additional pressure upon the solution which is required to cause the migration of as many molecules of water from the side of the solution to the side of the pure solvent as migrate simultaneously from pure water to the solution. Van't Hoff's theory demands that this pressure should increase with the number of molecules in the solvent and that it should be equal to the gas pressure of the solute in the volume of the solution. The actual measurements of osmotic pressure of solutions of cane sugar made by Morse[1] and his collaborators as well as by the Earl of Berkeley[2] and his fellow workers show a rather close approximation to van't Hoff's theory.

When the solution is separated by a strictly semipermeable membrane, the difference in the rate of diffusion of water in opposite directions must be the greatest at the beginning of the experiment, and the difference must diminish steadily during the experiment as a consequence of the increase of hydrostatic pressure on the side of the solution. The value of the osmotic pressure of a solution as defined above must therefore vary with the difference in the rate of the diffusion of water molecules in the two opposite directions at the beginning of the experiment. When we double the concentration of the solute we also double the initial difference in the rate of diffusion of water molecules in opposite directions, and the additional pressure

[1] Morse, H. N., The osmotic pressure of aqueous solutions, *Carnegie Institution of Washington, Publication 198*, 1914.

[2] Earl of Berkeley, and Hartley, E. G. J., *Proc. Roy. Soc. London, Series A*, 1916, xcii, 477.

which is to be applied to the solution to make the rate of diffusion of water in both directions equal must also be doubled.

This influence of the concentration holds strictly only as long as the solute influences the rate of diffusion of water simply by the number of its molecules (*e.g.* in preventing a number of water molecules from impinging on the solution side of the membrane this number being equal to the number of molecules of solute impinging during the same time). Van't Hoff's law, however, must become inadequate if the molecules of the solute can modify the rate of the diffusion of water by other forces than mere gas pressure; *e.g.*, by electrical forces varying with the nature of the molecule. This is probably true for any solute but in a much smaller degree when the solute is a non-electrolyte than when it is an electrolyte; and in the case of electrolytes it holds in a smaller degree when the electrostatic field around the individual oppositely charged ions is nearly the same (*e.g.* in the case of NaCl) than when it is very different as in the case of $Na_4Fe(CN)_6$ or $LaCl_3$. This influence of the electrical field surrounding the ions upon the rate of diffusion is due to the electrification of the water molecules.

We use the term electrification of water merely as a short expression of the fact that electrostatic forces cause water to migrate in a definite sense through a membrane. Whether this electrification of water particles is due to a cluster formation of water molecules around an ion as a nucleus, or to some other cause, may for the present remain outside the discussion.

It is the purpose of this paper to show that the electrification of water molecules by ions in solution must in certain cases result in a deviation of the osmotic pressure of a solution from that to be expected on the basis of van't Hoff's theory; and that the sense and relative quantity of deviation can be predicted.

The writer has recently investigated the influence of various ions on the rate of diffusion of water through a collodion membrane separating pure water from a watery solution. Before being used for the experiment the collodion membranes were filled over night with a 1 per cent solution of gelatin and kept in water. The next day the gelatin solution was removed and the collodion flasks were rinsed a considerable number of times with warm water to remove

the remnants of the solution. The initial treatment of the membranes with gelatin apparently modified the collodion permanently since after weeks of daily use these membranes, treated once with gelatin, behaved differently from membranes not treated with gelatin. We shall return to this fact in another publication. An investigation of the influence of electrolytes on the rate of diffusion of water through such collodion membranes previously treated with gelatin has shown that all the phenomena observed can be explained on the basis of the following two rules.

"1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion and diminishing inversely with a quantity which we will designate arbitrarily as the "radius" of the ion. The same rule applies to solutions of alkalies.

2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte. Solutions of acids obey the same rule."[3]

These two rules allow us to predict in which sense the *nature* of the electrolyte in solution should modify the osmotic pressure of a solution calculated on the basis of van't Hoff's law. Suppose pure water is separated by a collodion membrane from a watery solution of an electrolyte. When the electrolyte is one of those mentioned in Rule 1, *i.e.* possessing a monovalent or bivalent cation, water is attracted by the solution and diffuses from the side of pure water into the solution, as if the particles of water were positively charged. They should therefore be attracted by the anion and repelled by the cation of the electrolyte and the more so the higher the valency of these ions. Hence water should diffuse more slowly into a solution of M/192 $CaCl_2$ than into a solution of M/128 NaCl, and more slowly into M/128 NaCl than into M/192 Na_2SO_4, and considerably more slowly into M/128 NaCl than into M/256 Na_3PO_4 or M/320 $Na_4Fe(CN)_6$. If, however, the water particles are negatively charged everything is reversed, the water diffusing more rapidly into M/192 $CaCl_2$ than

[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 720.

into M/128 NaCl, and more rapidly into M/128 NaCl than into M/192 Na_2SO_4.

From the theoretical connection between the relative rate of diffusion of water from pure water to the solution through a semipermeable membrane discussed before, it follows that the osmotic pressure of a solution should be modified by the *nature* of the ions it contains in the same sense as the initial rate of diffusion of water is modified. This idea can be put to a test by the choice of electrolytes for which the collodion membrane is strictly semipermeable; *e.g.*, gelatin salts. Gelatin solutions attain in bags of collodion an osmotic pressure which is permanent, provided the hydrogen ion concentration of the solution does not undergo any change during the experiment.

II. Analogies between Solutions of Gelatin and Aluminium Salts.

Gelatin is an amphoteric electrolyte which when the hydrogen ion concentration of its solution exceeds the critical value 2×10^{-5} N forms only salts of the type of gelatin chloride, gelatin sulfate, etc., while when its hydrogen ion concentration falls below this value it can form only salts of the form of metal gelatinates; *e.g.*, Na gelatinate, Ca gelatinate, and so on. At the critical hydrogen ion concentration 2×10^{-5} N—the isoelectric point for gelatin—it can exist only in the form of pure, *i.e.* non-ionogenic, gelatin.[4] In this condition gelatin is practically insoluble, practically non-ionized, and is practically incapable of producing any osmotic pressure. Both types of gelatin salts, metal gelatinates as well as gelatin chloride, etc., are very soluble, are strongly ionized, and are capable of producing osmotic pressure. The writer's experiments, which are not yet all published, have shown that for each given hydrogen ion concentration there exists a definite equilibrium between non-ionogenic gelatin, gelatin salt, and free acid. If we have 1 per cent solutions of isoelectric gelatin the ionized or salt portion of the gelatin is practically zero. When we add increasing quantities of an acid, *e.g.* HCl, an increasing portion of the gelatin is transformed into gelatin chloride, while the portion of non-ionogenic gelatin is correspondingly dimin-

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.

ished. The relative proportion of gelatin salt and non-ionogenic gelatin depends therefore upon the hydrogen ion concentration of the solution and increases in a characteristic way with this concentration. This hydrogen ion concentration of the solution is, of course, not identical with the concentration of the acid added to the solution, since part of this acid is in combination with the gelatin forming the gelatin salt. Since practically only that fraction of the 1 per cent gelatin solution which is transformed into a gelatin salt produces an osmotic pressure it is obvious that the osmotic pressure of a 1 per cent solution of gelatin must change in a definite way with the hydrogen ion concentration of the solution. If we use different acids we find that different quantities of acid are required to bring a 1 per cent gelatin solution to the same pH.

When the hydrogen ion concentration is below the critical value 2×10^{-5} (or to use Sörensen's logarithmic symbol, when pH is > 4.7) a part of the non-ionogenic gelatin is transformed into metal gelatinate and this part is the greater the more the hydrogen ion concentration falls below 2×10^{-5}. There is again a definite equilibrium between hydrogen ion concentration, gelatin salt, and non-ionogenic gelatin.

Hence if we wish to compare the osmotic behavior of different gelatin salts we must see that the solutions have not only the same concentration of gelatin but also the same hydrogen ion concentration.[5]

The influence of solutions of gelatin on the electrification of water is the same as that of any other electrolyte and follows the two rules mentioned above. In solutions of metal gelatinates with monovalent or bivalent cations, water migrates through a collodion membrane as if the molecules of water were positively charged; in solutions of gelatin acid salts water migrates as if its particles were negatively charged. The turning point for the sense of migration seems to be near (or identical with) the isoelectric point of gelatin; *i.e.*, pH = 4.7.

[5] This latter point has been overlooked by the majority of colloid chemists who refuse to admit the chemical nature of the equilibrium between colloid and crystalloid, and who consequently ignore the rôle of the hydrogen ion concentration of the solution. Instead they compare the effect of the addition of equal quantities of different acids, overlooking the fact that they are thus comparing solutions in which the proportion of non-ionogenic gelatin and gelatin salt is different in the case of each acid.

It is perhaps not without interest that the behavior of gelatin is paralleled by the behavior of amphoteric electrolytes of a crystalloid character; *e.g.*, aluminium salts. Thus aluminium chloride exists only when the hydrogen ion concentration exceeds a certain critical value which seems to lie near that of the point of neutrality. When the solution becomes alkaline metal aluminates are formed. Na aluminate as well as $AlCl_3$ is very soluble. At the isoelectric point neither salt can exist and the insoluble $Al(OH)_3$ is formed.[6] The insoluble $Al(OH)_3$ has no osmotic pressure (or does not attract water) while solutions of both $AlCl_3$ as well as $NaAlO_2$ attract water powerfully. In the presence of $AlCl_3$ water molecules are apparently negatively electrified and in the presence of $NaAlO_2$ water shows positive electrification. The turning point for the sense of migration of water molecules seems to lie near or at the isoelectric point of aluminium; namely, pH about 7.0. It would be very important if we could measure the permanent osmotic pressure of aluminium salts in collodion bags, but this is impossible since aluminium salts (with the exception of the insoluble $Al(OH)_3$) diffuse through collodion membranes. It is, however, possible to determine the influence of different aluminium salts upon the rate of diffusion of water through a collodion membrane and it is found that this influence obeys the two rules.

We have mentioned this analogy between an amphoteric crystalloid, $AlCl_3$ and $NaAlO_2$ on the one hand, and an amphoteric colloid, gelatin chloride and sodium gelatinate, on the other to show that the fact of diffusibility or non-diffusibility through a collodion membrane does not force us to assume that the gelatin salts form no true solutions.

III. Osmotic Pressure of Different Metal Gelatinates.

Only the metal gelatinates with monovalent and bivalent cations need to be considered since the gelatin salts with trivalent cation seem to be insoluble. Different metal gelatinates were prepared

[6] The sparingly soluble $Al(OH)_3$ may be called a colloid since it does not diffuse through parchment paper. Sodium aluminate and $AlCl_3$ are crystalloids since they diffuse rapidly through such a membrane.

from gelatin rendered isoelectric (in the way described in former papers) by adding LiOH, NaOH, etc., to the gelatin. About 18 cc. of 0.01 N NaOH or $Ca(OH)_2$ must be contained in 100 cc. of a 1 per cent solution of isoelectric gelatin to obtain a metal gelatinate with a pH of 7.0; *i.e.*, with neutral reaction. We shall call a solution containing 1 gm. of isoelectric gelatin in 100 cc. a 1 per cent gelatin solution notwithstanding the fact that the gelatin is caused to combine with an acid or base.

We will first show that the gelatin salts with monovalent and bivalent cations influence the rate of diffusion of water in the same sense as is done by common crystalloid salts with monovalent and bivalent cations. Since in the presence of such metal gelatinate water diffuses through a collodion membrane as if its particles were positively charged (as ascertained by experiments with a constant current) we should expect that water should diffuse more rapidly into 1 per cent solution of metal gelatinate with monovalent cations, Li, Na, K, NH_4, than with bivalent cations, Mg, Ca, Sr, Ba, etc. This is indeed the case. We proceeded in the same way as in our previous experiments. Collodion bags,[7] in the shape of Erlenmeyer flasks with about 50 cc. contents, and all with the same surface, were prepared in as uniform a way as possible. The flasks were closed with a perforated rubber stopper through the opening of which a glass tube (with bore of about 2 mm. diameter) serving as a manometer was pushed. These collodion flasks were filled with water distilled in a tin still and having a pH of about 5.2. The collodion bag was put into a beaker filled with a 1 per cent solution of a metal gelatinate and the pressure head of the water in the manometer tube was at the beginning of the experiment about 120 mm. of H_2O. The fall of the level of the water in the glass tube was measured in definite intervals. In Figs. 1 and 2 the levels are plotted as ordinates over the time elapsed since the beginning of the experiment. The experi-

[7] The permeability of the collodion bags was tested before each experiment by filling them with M/4 cane sugar and measuring the rate at which water diffused into them. When the water rose in the glass tube with a bore of 2 mm. in diameter to a height of about 110 mm. in 20 minutes the membranes were considered serviceable.

ments were made at a constant temperature of 24°. The two figures show that water diffuses more rapidly into Li and K gelatinate than into Ba and Ca gelatinate. Na and NH_4 gelatinate behaved like Li and K gelatinate. The pH of the gelatin solutions was 8.2 in this experiment.

A second method of testing the influence of different metal gelatinates upon the rate of diffusion consisted in determining that concentration which a cane sugar solution must have in order to balance

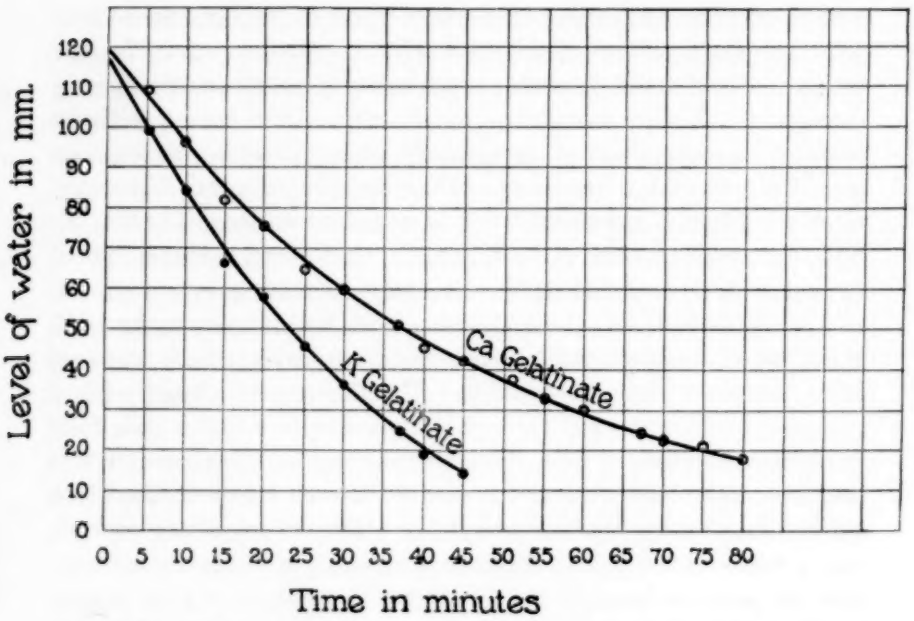

FIG. 1. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solutions of Ca and K gelatinate (pH = 8.2).

the attraction of a 1 per cent solution of metal gelatinate for water. The procedure was as follows. 350 cc. of a 1 per cent solution of a metal gelatinate, *e.g.* Na gelatinate, of pH 7.0 were put into each of a series of beakers and into each beaker was put one of the collodion flasks filled with a different concentration of cane sugar varying from

M/1 to M/64. It was useless to go below a concentration of M/64 of cane sugar since this was about the lowest concentration at which cane sugar influenced the rate of diffusion of water. The level of the cane sugar solution in the manometer tube was about 25 mm. at the beginning of the experiment. When the water diffused more rapidly from gelatin to cane sugar than in the opposite direction, the level in the manometer rose, when the water diffused more rapidly

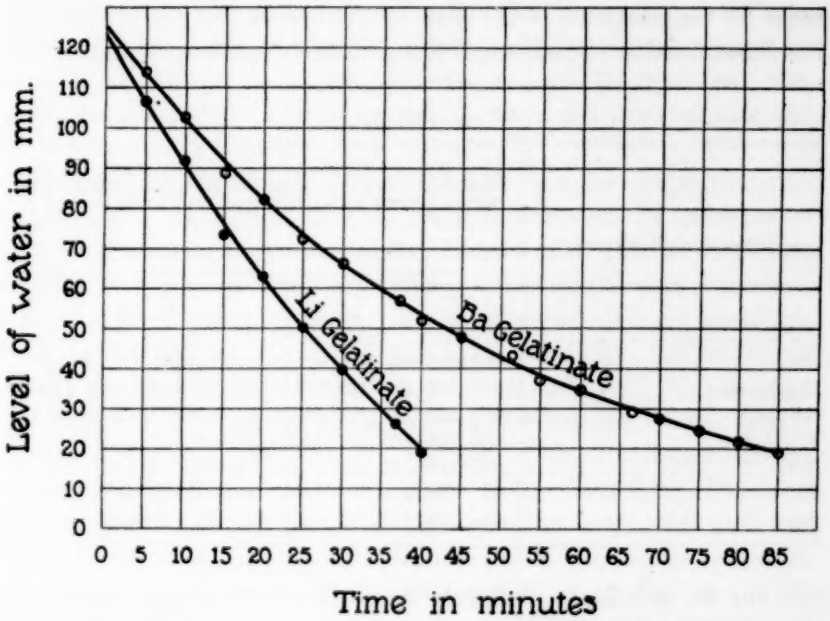

FIG. 2. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solution of Ba and Li gelatinate (pH = 8.2).

from cane sugar into gelatin than in the opposite direction the level in the manometer tube fell. Between the two was a concentration where the rate of diffusion in both directions was the same and this concentration of cane sugar we called the balancing concentration of cane sugar. Such experiments are only of value when of short duration on account of the fact that the sugar diffuses out into the gelatin

solution. In Table I the level of the water in the manometer tube after 30 minutes is given for the different 1 per cent gelatin solutions. The plus sign means a rise in the level of water in the cane sugar solution above the original level of 25 mm., the minus sign means a fall of the level of the sugar solution in the manometer tube.

We notice that the concentration which a cane sugar solution must have to balance osmotically 1 per cent solutions of gelatin salts with monovalent cations of a pH of about 7.0 lies between M/8 and 3M/32, while for Ca gelatinate of the same concentration and pH the balancing concentration of cane sugar has a value between M/32 and M/16,

TABLE I.

Nature of gelatin salt used.	Change of level of liquid in the manometer tube of different concentrations of cane sugar solution when immersed in 1 per cent solutions of different metal gelatinates of pH 7.0, after 30 min.										
	Concentration of cane sugar solution.										
	M/1	3M/4	M/2	3M/8	M/4	3M/16	M/8	3M/32	M/16	M/32	M/64
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Li gelatinate.....	358	294	195	109	+60	+24	−4	−11	−16	−21	−16
Na "	397	289	251	120	+97	+22	−3	−11	−20	−19	−17
K "	379	285	186	102	+62	+22	−3	−7	−15	−18	−22
NH_4 "	390	323	222	127	+96	+35	+4	−4	−16	−19	−20
Ca "	397	300	241	172	+99	+69	+33	+16	+2	−2	−18
Ba "	376	310	225	145	+97	+77	+36	+29	+12	0	−15

and for Ba gelatinate a slightly lower value. We may, therefore, state that the balancing concentration of cane sugar is roughly over twice as great when the metal gelatinate has a monovalent cation as when it has a bivalent cation. Since water diffuses towards a cane sugar solution with a velocity which in the beginning of the experiment increases in proportion with the concentration of the sugar, we can say that the rate of diffusion of water into 1 per cent solutions of gelatin salts of the type Na gelatinate is between two and three times as great as the rate of diffusion of water into a solution of Ca or Ba gelatinate of the same concentration and pH.

The question now arises; Is there a similar difference between the

permanent osmotic pressure of 1 per cent solution of metal gelatinate when the cation is monovalent and bivalent? We have already published such measurements before we were aware of the fact that the electrification of water might play a rôle in osmotic pressure. In a paper published in this *Journal*[8] we have shown that when 1 per cent solutions of gelatin salts with monovalent cation—Li, Na, K, and NH_4 gelatinate—are separated by collodion membranes from distilled water they may reach a maximal osmotic pressure of from 300 to 325 mm. of gelatin solution, while the 1 per cent gelatin solutions with bivalent cation—Ca and Ba gelatinate—never reach an osmotic pressure higher than 130 mm.; *i.e.*, a little more than one-third the value of the highest osmotic pressure of 1 per cent solutions of gelatin salts with monovalent cation.

It seemed desirable to get the time curve for the rise in pressure, since such a curve permits a comparison between the influence of the cation on both the velocity of the diffusion of water from water into solution as well as on the pressure when osmotic equilibrium is reached. The experiment was as follows. The gelatin was rendered isoelectric in a way described in a previous paper. To 1 gm. of isoelectric gelatin were added 20 cc. of 0.01 N of an alkali (LiOH, NaOH, etc.) and then enough distilled water to bring the solution of the gelatin (which was melted) to 100 cc. The pH of these solutions in the experiment under discussion turned out to be about 8.8. The solutions were put into the collodion flasks and the latter were put into beakers containing 350 cc. of H_2O, the pH of which was raised at the beginning to about 9.0 by adding 0.2 cc. of 0.01 N NaOH to 350 cc. of H_2O, in order to prevent a rapid lowering of the pH of the gelatin solution through the CO_2 absorbed from the air by the distilled water of the outside solution. The temperature was 24°C. The pH decreased slowly in the beaker and as a consequence also in the gelatin solution, and in 20 hours the pH had fallen to about 7.0 in the gelatin solutions with monovalent cations, while it had fallen less in the gelatin solutions with bivalent cations. When the pH fell, the osmotic pressure also began to diminish on account of the shifting of

[8] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483.

the equilibrium between non-ionogenic gelatin and the metal gelatinate formed.[9]

Fig. 3 gives the rise of level of liquid in the manometer during the first 12 hours for a 1 per cent NH_4 gelatinate and a 1 per cent Ba gelatinate solution. Equilibrium was reached after about 6½ hours. The slight diminution of osmotic pressure due to the fall of pH on

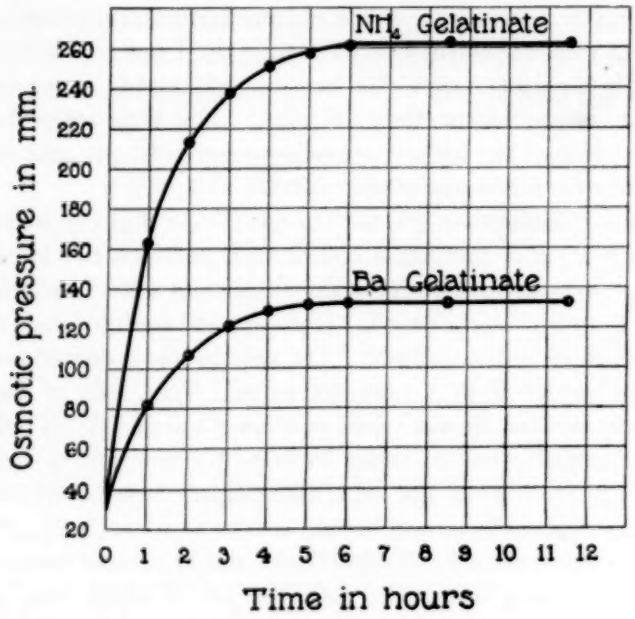

FIG. 3. Curves of rise of osmotic pressure of 1 per cent solutions of NH_4 and Ba gelatinate (pH = 8.8). Pressure in mm. of column of gelatin solution.

account of CO_2 action did not commence until later. The osmotic pressure of the 1 per cent solution of NH_4 gelatinate is in this experi-

[9] In order to make sure that the osmotic pressure reached is permanent, the experiments with metal gelatinates should be made with the exclusion of CO_2. Since, however, this source of error does not exist in the case of gelatin acid salts where the permanency of the final pressure reached can be made sure of, and since the results in that case are the same, as we shall presently see, and since the error was less for Ca and Ba gelatinate than for Na gelatinate, we need not dwell upon this point.

ment about twice as high as that of the 1 per cent solution of Ba gelatinate of about the same pH. During the first hour the relative rise of level in the solutions is also approximately as 1:2. Fig. 4 gives the curves for Na gelatinate and Ca gelatinate. The curve for Na gelatinate in Fig. 4 is identical with the curve for NH_4 gelatinate in Fig. 3 and the curve for Ca gelatinate in Fig. 4 is identical with the curve for Ba gelatinate in Fig. 3. The curves for Li and K gelatinate were identical with the curves for NH_4 and Na gelatinate in Figs. 3 and 4. Such results are always obtainable when both the

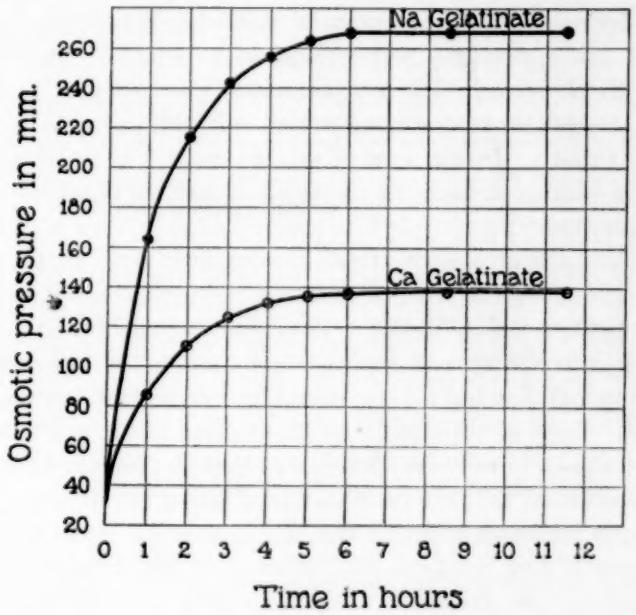

FIG. 4. Curves of osmotic pressure of 1 per cent solutions of Na and Ca gelatinate (pH = 8.8). Pressure in mm. of column of gelatin solution.

concentration and the pH of the solutions are identical. We therefore reach the conclusion that the different metal ions influence the osmotic pressure of gelatin solutions in the same sense as they influence the rate of migration of water into the solution. This supports the idea that the electrification of the water particles plays a rôle in the magnitude of the osmotic pressure as obtained by the use of semipermeable membranes.

IV. The Gelatin Cation.

When the hydrogen ion concentration of the gelatin solution is higher than 2×10^{-5} the gelatin can exist only in the form of gelatin chloride, sulfate, citrate, etc. Water is electrified negatively by such gelatin solutions and hence when pure water is separated by a collodion membrane from a 1 per cent solution of gelatin chloride or sulfate, etc., the water should diffuse more rapidly into the gelatin solution when the anion is monovalent than when it is bivalent. We have shown in a former paper[10] that all dibasic and tribasic acids (thus far tried by us), with the exception of sulfuric acid, combine in molecular and not equivalent proportions with gelatin. Thus gelatin forms with phosphoric acid a monogelatin phosphate, with tartaric acid a monogelatin tartrate; only with sulfuric acid does it form a digelatin sulfate. In the case of gelatin oxalate it is possible that we have a mixture of both the monogelatin and the digelatin oxalate, the former prevailing.

This fact is of great importance for our problem. In the case of monogelatin phosphate the anion is not trivalent PO_4, but essentially the monovalent anion H_2PO_4. The same is true for monogelatin citrate and in the case of monogelatin oxalate the anion is not a bivalent oxalate ion but a monovalent oxalate ion with one hydrogen attached. Only in the case of gelatin sulfate is the anion divalent. We have shown in our preceding paper that in the case of NaH_2PO_4 the attraction of the salt for water is of the order of magnitude of a salt with monovalent anion like NaCl, showing that the presence of the two H ions weakens the influence of the trivalent PO_4 ions considerably. We therefore should expect that in the case of salts like monogelatin citrate, monogelatin phosphate, monogelatin oxalate, and monogelatin tartrate the influence of anion on the rate of the diffusion of water should be of the same order of magnitude as in the case of gelatin chloride or nitrate; while in the case of digelatin sulfate the SO_4 ion should act as a divalent anion producing a stronger repelling effect on the negatively charged water than is done by gelatin chloride. This turns out as expected (Fig. 5).

[10] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 559.

When we fill the collodion bags as described with distilled water and put the bags into 1 per cent solutions of different gelatin acid salts of the same pH, giving the water inside the bag an initial pressure head of about 125 mm. of H_2O, the water will diffuse out more slowly against the gelatin sulfate than against any of the solutions of the other gelatin salts. This is demonstrated by Fig. 5. The abscissæ are the time in minutes, the ordinates the level of water in

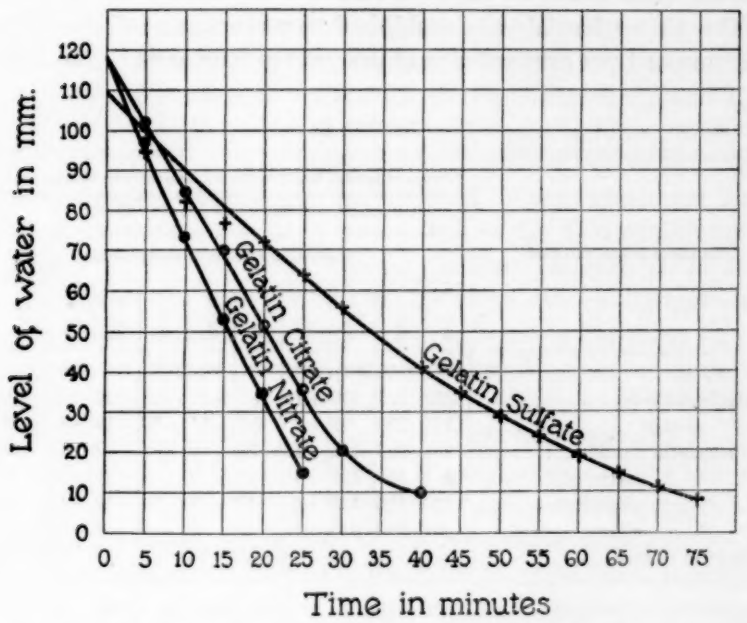

FIG. 5. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solutions of gelatin sulfate, gelatin citrate, and gelatin nitrate (pH = 3.5).

the manometer of the collodion flask. The initial pressure head was 125 mm. It required about 25 or 30 minutes for the pressure head of distilled water to fall from 120 mm. to about 20 mm. when it diffused into gelatin nitrate and monogelatin citrate, and 60 minutes when the distilled water diffused into gelatin sulfate.

The second method of testing the influence of the anion on the rate of diffusion of water consisted in determining the balancing

concentration of cane sugar for each of these salts in the way described. Table II gives the values observed after 30 minutes. The gelatin solutions had a pH of 3.5.

Using the same criterion as before we find that the balancing concentration of cane sugar lies for gelatin sulfate between 3M/32 and M/8 and for all the others between 3M/16 and M/4. In other words, the balancing concentration possesses for gelatin sulfate about one-half the value found for the other salts.

The writer has already published measurements of the osmotic pressure of 1 per cent solution of different gelatin salts. The osmotic

TABLE II.

Nature of gelatin salt used.	Change of level of liquid in the manometer tube of different concentrations of cane sugar solution when immersed in 1 per cent solutions of gelatin acid salts of pH 3.5, after 30 min.										
	Concentration of cane sugar solution.										
	M/1	3M/4	M/2	3M/8	M/4	3M/16	M/8	3M/32	M/16	M/32	M/64
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Gelatin chloride	265	198	110	+15	+6	−18	−12	−22	−22	−19	−19
" nitrate	262	200	98	+25	+8	−11	−10	−19	−18	−19	−16
Monogelatin oxalate	273	203	146	+75	+33	0	−4	−12	−17	−15	−20
" tartrate	288	215	159	+47	+30	−4	−6	−20	−14	−16	−19
" phosphate	290	196	125	+45	+18	−8	−14	−19	−14	−13	−16
" citrate	335	244	115	+53	+30	0	−5	−9	−16	−24	−11
Digelatin sulfate	278	215	143	+74	+35	+10	+2	−8	−14	−14	−19

pressure varies for the same concentration of a gelatin salt with the pH of the solution and is a maximum at a pH of about 3.4. It was found that the maximum osmotic pressure of a 1 per cent solution of gelatin chloride, gelatin bromide, gelatin nitrate, gelatin acetate, monogelatin oxalate, monogelatin tartrate, monogelatin phosphate, and monogelatin citrate is about 320 mm. of the gelatin solution; while for a 1 per cent solution of gelatin sulfate the highest pressure obtainable is about 130 mm. This is in satisfactory agreement with the ratio we should expect on the basis of the influence of the anions on the rate of diffusion of the negatively electrified particles of water.

We considered it necessary to obtain also the curves for the increase

in the height of the level of water in the manometer with time until the equilibrium is reached. 1 per cent solutions of gelatin chloride, nitrate, oxalate, tartrate, citrate, and sulfate were prepared by making up the solution with the addition of as much of these acids to 1 gm. isoelectric gelatin as was required to produce a pH of 3.5. These 1 per cent gelatin solutions were put into collodion flasks containing the glass tube as described and each flask was put into a beaker with 350 cc. of distilled water the pH of which was made 3.0 by adding in each case the same acid as that of the gelatin. In Fig. 6 is plotted the rise and the final osmotic pressure of these solutions. The osmotic pressure reached in the case of gelatin sulfate was about 159 mm. while for the other salts it was almost twice as high (between 260 and 310 mm.). It is also noticeable from the curves that the relative velocity of rise during the 1st hour of the experiment also showed about the same ratio of almost 1:2 as the final equilibrium. The results thus confirm our expectation that on account of the electrification of the water molecules both the rate of diffusion as well as the final osmotic equilibrium are affected in the same sense.

If we wish to explain the differences between the observed osmotic pressure of solutions of calcium and sodium gelatinate or of gelatin chloride and gelatin sulfate, exclusively on the basis of van't Hoff's law, we are compelled to seek refuge in the assumption of the formation of aggregates of gelatin ions by which the number of these particles is diminished without diminution of the number of their charges.[11] This suggestion was first offered by Bayliss[12] when he found that the osmotic pressure of the colloid Congo red was considerably lower than was to be expected according to the molecular concentration and the conductivity of the solution. This assumption, however, which the writer had also tentatively accepted to explain the difference between the osmotic pressure of calcium and sodium gelatinate of the same concentration rests only on the facts which the assumption is supposed to explain, while the connection between rate of diffusion of water and osmotic pressure of a solution holds generally in all

[11] The writer has shown that the conductivity of 1 per cent Na gelatinate and of 1 per cent Ca gelatinate at the same pH is identical.

[12] Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv. 229.

cases of dynamical equilibrium between two processes which occur simultaneously in opposite directions; *e.g.*, chemical equilibrium between reversible reactions.

Procter[13] and his collaborators have developed a theory of swelling of colloids based on the assumption that swelling is a purely osmotic phenomenon. On the basis of this theory we may consider the

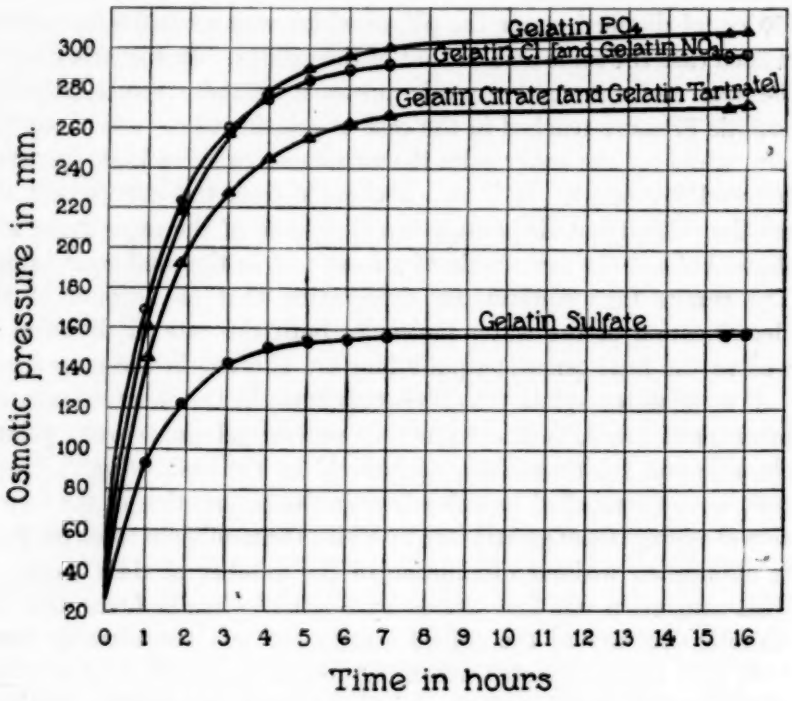

FIG. 6. Curves of osmotic pressure of 1 per cent solutions of gelatin phosphate, gelatin chloride (nitrate), gelatin citrate (tartrate), and gelatin sulfate (pH = 3.5). Pressure in mm. of column of gelatin solution.

surface and all the other solid parts of a block of gelatin to act as membranes permeable for water and crystalloids but not for gelatin. The osmotic conditions inside a solid block of gelatin which is

[13] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Procter, H. R., and Burton, D., *J. Soc. Chem. Ind.*, 1916, xxxv, 404.

submersed in water are therefore comparable to those in our experiments except that we substitute a collodion membrane for the gelatin membrane.

The writer has shown that metal gelatinates of the same concentration of gelatin and of hydrogen ions swell much more when the metal in combination with the gelatin is monovalent (*e.g.* Li, Na, K, or NH_4) than when it is bivalent (*e.g.* Ca or Ba).[8] When gelatin is a cation the swelling is greater when the anion in combination with gelatin is monovalent (*e.g.* Cl, NO_3, or H_2PO_4) than when it is bivalent (*e.g.* SO_4).[10] The curves representing the influence of different acids and alkalies on the swelling of gelatin are similar to the curves representing the influence of the same acids and alkalies on the osmotic pressure of gelatin solutions. This similarity becomes clear if we adopt Procter's osmotic theory of swelling, adding to it our theory of the rôle of the electrification of particles of water in the phenomena of osmosis. In these experiments the solution surrounding the block of gelatin salt must have a low concentration of electrolyte since the presence of an excess of electrolyte suppresses the swelling, as pointed out in previous publications.

SUMMARY.

1. Amphoteric electrolytes form salts with both acids and alkalies. It is shown for two amphoteric electrolytes, $Al(OH)_3$ and gelatin, that in the presence of an acid salt water diffuses through a collodion membrane into a solution of these substances as if its particles were negatively charged, while water diffuses into solutions of these electrolytes, when they exist as monovalent or bivalent metal salts, as if the particles of water were positively charged. The turning point for the sign of the electrification of water seems to be near or to coincide with the isoelectric point of these two ampholytes which is a hydrogen ion concentration of about 2×10^{-5} N for gelatin and about 10^{-7} N for $Al(OH)_3$.

2. In conformity with the rules given in a preceding paper the apparently positively charged water diffuses with less rapidity through a collodion membrane into a solution of Ca and Ba gelatinate than into a solution of Li, Na, K, or NH_4 gelatinate of the same concentra-

tion of gelatin and of hydrogen ions. Apparently negatively charged water diffuses also with less rapidity through a collodion membrane into a solution of gelatin sulfate than into a solution of gelatin chloride or nitrate of the same concentration of gelatin and of hydrogen ions.

3. If we define osmotic pressure as that additional pressure upon the solution required to cause as many molecules of water to diffuse from solution to the pure water as diffuse simultaneously in the opposite direction through the membrane, it follows that the osmotic pressure cannot depend only on the concentration of the solute but must depend also on the electrostatic effects of the ions present and that the influence of ions on the osmotic pressure must be the same as that on the initial velocity of diffusion. This assumption was put to a test in experiments with gelatin salts for which a collodion membrane is strictly semipermeable and the tests confirmed the expectation.